Characterisation and aetiology of Jelly-like gonad condition (JGC) in the common carp, *Cyprinus carpio* (L)

By
Raihan Mahmud

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Institute for Marine and Antarctic Studies (IMAS)
University of Tasmania
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Declaration of Originality

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This thesis comprises non-published contents. The following people and institutions contributed to the work undertaken as part of this thesis:

Signed:

Raihan Mahmud, Institute of Marine and Antarctic Studies, University of Tasmania (Candidate)

Signed:

Dr. Jawahar Patil, Institute of Marine and Antarctic Studies, (Primary supervisor)

Signed:

Professor Chris Carter, Head of the Fisheries and Aquaculture Centre, Institute of Marine and Antarctic Studies (supervisor)

Signed:

Associate professor John Purser, Former Deputy Head, Fisheries and Aquaculture Centre and Deputy Associate Dean International for IMAS (supervisor)

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Thesis abstract

Fisheries is the fifth largest food production industry, worth more than $2.2 billion to the Australian economy every year. However, the aquatic ecosystems that support these fisheries are very vulnerable to threats such as invasion by aquatic pest species, which can negatively impact native biodiversity and increase risks to allied industries such as recreational fisheries and aquaculture. The common carp (*Cyprinus carpio*) is extensively farmed in Asia, as well as in Europe and Middle East. Whilst it is a popular angling fish in Europe, it is considered a significant pest in many countries including North America, Canada, New Zealand and Australia. Overall, carp cause significant impact on aquatic ecosystems by increasing turbidity, changing nutrient composition, altering the macrophyte and macroinvertebrate composition and can quickly become the dominant species after introduction. Immediate control of carp in Australia is required as its distribution has exceeded an area of one million km^2. As a species with high reproductive capability, both aquaculture and pest management of carp could benefit from greater knowledge about the control of reproductive capability. This study aimed to examine a naturally occurring 'sterile' condition observed during the routine carp management program in Lake Sorell, Tasmania, Australia where the fish develops a testicular condition without any external symptoms. Occurrence of gonad abnormality in wild fish is rare but has been reported in a number of species including carp. However, the gonadal condition observed in Lake Sorell has many unique characteristics that make it distinguishable from all of the previously studied cases. Morphologically the affected gonad appears 'jelly like' and therefore described as Jelly-like Gonad Condition (JGC). The overarching objective of the study was to describe the JGC condition, specifically: 1) determine its characteristics, including prevalence, morphology and histopathology; 2) test relatedness of the condition to select biotic and abiotic factors, and; 3) investigate the expression signature to discern a genetic role in its development.

Objectives were addressed by analyses of catch data, gross morphology, histology, milt quality, hormone levels and next generation total transcriptome profiling of JGC and normal carp testes. Gross examination of the gonad revealed multi-nodular fluid filled, clear or semi opaque blisters in JGC affected testicular tissue. Investigation of over 6000 fish indicated the JGC is male specific i.e. it does not occur in female gonadal tissue, with increasing frequency that was related to maturity of the fish. Parallel examinations in the golden galaxiid, short fin eel and brown trout that inhabit the same lacustrine system indicate that the condition is absent in all three species, suggesting a species specificity of the condition. Morphological analysis using ImageJ suggested four distinguishable severity stages—early (stage 1), mild (stage 2), medium (stage 3) and severe (stage 4). Additionally, histology with differential and fluorescent TUNEL staining for each severity stage revealed the early stages (stages 1 and 2) of JGC were marked by an abnormally high number of Sertoli cells (11 fold) coinciding with arrest of spermatogenesis whereas late stages were marked by apoptotic cells. Collectively, data suggested that this fish could be an excellent model for understanding Sertoli cell malignancy and testicular cell apoptosis.

Regression modelling using 4,594 fish caught between 2013-2017 indicated a significant relationship of the JGC occurrence to body weight, fork length and fishing year. One-way ANOVA followed by Tukey's HSD was used to confirm the accelerated growth of JGC fish compared to normal male, female and immature fish. However, no relationship of the condition was associated with month of capture, fish strain (i.e. scale type) or capture location. Growth analysis of mark-recaptured fish indicated that this enhanced growth is triggered post onset of JGC. Quantitative and qualitative assessment of JGC and normal milt using spermatocrit and Bengal rose staining followed by light and phase contrast microscopy was undertaken. Briefly, quantity and quality of spermatozoa dropped drastically with the advancing condition. Significantly, a higher number of

sperm abnormalities (e.g. coiled tail sperm, broken tail sperm) was associated with advanced JGC. Similarly, analysis of the normal and JGC milt using computer assisted sperm analysis (CASA) indicated that motility, activity time and velocity parameters (e.g. VCL, VAP, VSL) drop significantly in the advanced condition. Collectively data suggested that JGC fish are near sterile, particularly in the advanced conditions. Hormonal analysis using ELISA confirmed the level of circulating 11-Keto-testosterone (11-KT) in JGC fish was significantly ($p<0.05$) lower than in unaffected males. However, the Luteinising hormone (LH) level in primary JGC fish (Stage 1-3) was similar to unaffected males and females, yet significantly higher in Stage 4. While a lower level of 11-KT supports the dysfunctionality of the testis, a higher level of LH in extremely affected JGC fish indicates the feedback regulation of LH by 11-KT.

Transcriptome analyses using next generation total RNA sequencing (RNAseq) followed by differential expression analysis revealed that 7,129 genes are differentially expressed between JGC and control testis, of which 40 genes were found to have central roles in development and/ or regulation of the condition. BLAST analyses of the 40 candidate genes indicated that many (n=26) of these are novel to date. Pathway analysis of the differentially expressed genes revealed that 130 pathways including chromosome separation, cell morphogenesis, nucleic acid activity, spermatogenesis, gonad development, steroid production and apoptosis were significantly altered in the JGC testis. However, no immune pathway/s were enriched. Collectively, RNAseq analysis followed by the pathway investigation of the differentially expressed genes discovered the key pathways that are responsible for the large cellular changes in JGC tissue observed in histology.

It appears that this sterile condition is unique but shares key features with human non-germ cell testicular cancer. For example, the initial uncontrolled proliferation of Sertoli

cells with upregulation of many cancer markers (e.g. cx43, fbox32, sall4) suggests that the condition is likely triggered by a malignant pathway. Observed non-activation of immune system process with microbiological investigation supports the hypothesis of non-pathogenic origin. Although, few cases of Sertoli cell malignancy have been reported previously, observed mass apoptosis of all testicular cell resulting in a sterile animal is unique making JGC carp a potential model to study testicular non-germ cell cancer, apoptosis, sterility and related reproductive processes. Moreover, high prevalence of this naturally occurring condition should permit comparative evaluation between individuals allowing rigorous testing, including therapies.

In conclusion, this study suggests that JGC carp are near sterile and exhibit enhanced somatic growth compared with unaffected cohorts. JGC carp can be used as sterile Judas fish in carp control programs such as the one in Tasmania and may have excellent potential for use in aquaculture. Moreover, future characterization of the 'novel' genes (n=26) associated with the condition may lead to the discovery of unique master regulator of testicular cancer, Sertoli cell specific marker, sterility or fertility that are highly relevant to human health. The sterility associated gene/s could also provide opportunities for developing species specific genetic carp eradication options.

List of abbreviations

General

ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
CASA	Computer Assisted Sperm Analysis
DEG	Differentially Expressed Gene
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked Immunosorbent Assay
EtOH	Ethanol
FDR	False discovery rate
GnRH	Gonadotropin Releasing Hormone
H&E	Haematoxylin and Eosin
IFS	Inland Fisheries Service
JGC	Jelly like Gonad Condition
lncRNA	Long non-coding RNA
NBF	Neutral Buffered Formalin
NCBI	National Center for Biotechnology Information
ncRNA	Non-coding RNA
PBS	Phosphate Buffered Saline
QC	Quality control
RNA	Ribonucleic Acid
S.E.	Standard Error
µg	Microgram
µl	Microliter
ml	Milliliter

Gene Names

abtb2a	ankyrin repeat and BTB/POZ domain-containing protein 2
adcy6b	adenylate cyclase type 6
adcy9	adcy9 gene
anapc11	anaphase-promoting complex subunit 11
apoeb	apolipoprotein Eb
arnt2	aryl hydrocarbon receptor nuclear translocator 2
baiap2a	brain-specific angiogenesis inhibitor 1-associated protein 2
bcl2l1	bcl-2-like protein 11
boka	bcl-2-related ovarian killer protein homolog A
cacna1fb	voltage-dependent L-type calcium channel subunit alpha-1F
camk2b	protein kinase C beta type
capn2l	calpain-2 catalytic subunit
caprin2	caprin-2
ccdc103	coiled-coil domain containing 103
ccdc40	coiled-coil domain-containing protein 40
cdc26	anaphase-promoting complex subunit CDC26
cfap157	cilia- and flagella-associated protein 157
cfap20	cilia- and flagella-associated protein 20
cx43	gap junction alpha-1 protein
cxcl12a	stromal cell-derived factor 1
cyp26a1	cytochrome P450 26A1
dcdc2b	doublecortin domain-containing protein 2
dnaaf1	dynein axonemal assembly factor 1
dnase2	deoxyribonuclease-2
dner	delta and Notch-like epidermal growth factor-related receptor
dynlrb1	dynein light chain roadblock-type 1
faima	fas apoptotic inhibitory molecule 1-
fbln1	fibulin-1
fbxo32	F box protein 32
fgfr1a	fibroblast growth factor receptor 1-A
foxj1a	forkhead box protein J1-A
foxn4	forkhead box protein N4
fynb	tyrosine-protein kinase fynb
gsnb	gelsolin

hormad1	HORMA domain-containing protein 1
htra1b	serine protease HTRA1B
ift57	intraflagellar transport protein 57 homolog
itpr1b	inositol 1,4,5-trisphosphate receptor type 1
jun	transcription factor AP-1
klhl10a	kelch-like protein 10
klhl26	kelch-like protein 26
mei4	meiosis-specific protein MEI4-like
meig1	meiosis expressed gene 1 protein homolog
mmp14a	matrix metalloproteinase-14
mmp2	72 kDa type IV collagenase
mpp5a	MAGUK p55 subfamily member 5-A
muc5.1	mucin-2
ncs1a	neuronal calcium sensor 1
ndrg1a	protein NDRG1-like
pdgfd	platelet-derived growth factor D-like
pimr129	pollen-specific leucine-rich repeat extensin-like protein 1
pitx2	pituitary homeobox 2
ptk2ba	protein-tyrosine kinase 2-beta
rhoab	rho-related GTP-binding protein RhoA-B
rlim	E3 ubiquitin-protein ligase RLIM
rnf14	E3 ubiquitin-protein ligase RNF14-like isoform X1
ropn1l	ropporin-1-like protein
rsg1	REM2- and Rab-like small GTPase 1
scg2a	secretogranin-2
skor1b	SKI family transcriptional corepressor 1 homolog-B
sla1	src-like-adapter
socs2	suppressor of cytokine signaling 2
spire1b	protein spire homolog 1
sptbn1	Eukaryotic cytoskeleton proteins
stm	protein starmaker
tbx1	T-box transcription factor TBX1
tfap2d	ranscription factor AP-2 delta
tob1a	protein Tob1
triobpb	TRIO and F-actin-binding protein

tsga	testis specific 10
ttc25	tetratricopeptide repeat protein 25
ttc26	intraflagellar transport protein 56
vegfbb	snake venom vascular endothelial growth factor toxin barietin

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Chapter One: General Introduction

1.1 Common carp

Common carp (*Cyprinus carpio*), also known as European carp is a ubiquitous benthivore teleost belonging to the family of Cyprinidae. Common carp is native to Black, Caspian and Aral sea drainage (Balon, 1995) and considered as one of the oldest cultured species in the world (Balon, 2004; Hulata, 1995). Common carp has been domesticated for food and ornamental purposes (Amano, 1970; Edwards and Twomey, 1982; Li and Moyle, 1993) as well as being a popular recreational fish (Arlinghaus, 2007; Arlinghaus and Mehner, 2003). As a species with high environmental tolerance (Koehn and MacKenzie, 2004), common carp has been successfully introduced to all continents except Antarctica (Balon, 1995). Domestication of the species began in medieval times, although the purpose varied in different parts of the world. Through domestication, artificial selection and geographical isolation, numerous strains of carp have evolved over time.

1.1.1 Carp in aquaculture

Carp like many aquatic animals have been introduced to meet increasing aquaculture demand throughout the world. They are the third most widely cultivated and economically important finfish species in the world (FishStat, 2013). According to Food and Agriculture Organisation (FAO, 2002) farmed common carp comprise nearly 14 percent of the total aquaculture production around the world. While carp can be cultured in various aquaculture systems, semi-intensive pond polyculture in Asia and India (Rahman, 2015) and intensive culture in Europe are most commonly used (Carter, 2006). However, the extreme reproductive capability of carp often poses a threat during their extensive culture. For example, frequent reproduction of carp often results in higher stocking density than is desirable, especially for polyculture (Feldlite and Milstein, 2000; Jha and Barat, 2005). Additionally, early gonadal maturation diverts energy from their somatic growth, which results in a deterioration of flesh quality in fish (Zohar, 1989).

1.1.2 Carp as a pest species

Although carp has high economic and research potential, extreme ecological disruption on native biodiversity has led to this species being frequently reported as nuisance species in many countries like North America, Canada, New Zealand and Australia (Koehn, 2004; Koehn and MacKenzie, 2004). In particular, carp are well known for their ability to modify the habitat they are introduced to by sucking up mud and expelling fine sediment particles. This feeding behaviour not only uproots native aquatic vegetation but also increases water turbidity (Haynes, 2011). The resulting algal bloom produced from the transfer of nutrients from the sediment to the water column further impedes native fish populations (Breukelaar et al., 1994; Crivelli, 1981; Khan, 2003; Koehn, 2004; Neess et al., 1957; Newcombe and MacDonald, 1991; Wilcox and Hornbach, 1991).

Carp were introduced to Australia both deliberately and accidentally on multiple occasions (Brown, 1996; Davis et al., 1999), with at least three different population strains being recognised (Shearer and Mulley, 1978). Over the last 50 years, carp populations have increased tremendously and are now prevalent throughout south-eastern Australia (Koehn, 2004; Shearer and Mulley, 1978) including parts of Tasmania (Diggle et al., 2012). Populations introduced to Boolarra, Victoria in 1960 were subsequently translocated into the Murray-Darling system (Wharton, 1971), where they now comprise of up to 90% of the fish biomass in some locations within this water basin (Harris and Harris, 1997; Norris, 2011). The distribution of carp has exceeded over one million km^2 within Australia, stressing the need for immediate population control of this species (Koehn, 2004).

Carp were rediscovered in Tasmania in 1995 in the interconnected lakes Crescent and Sorell (Diggle et al., 2012; Donkers et al., 2012). An extensive eradication process began immediately to physically remove individuals, to contain the spread of carp and to prevent

further recruitment (Diggle et al., 2012; Yick, 2015). While the program was successful in Lake Crescent, eradication in Lake Sorell is still ongoing. The Lake Sorell population was also fished down significantly but for a recruitment event in 2009 that resulted in explosion of population (IFS 2010, 2012). However, due to concerted management measures, the population has been limited to a single year cohort (2009 Cohort), making this an interesting population model to study. As the Tasmanian carp originated from a handful of individuals that escaped from live bait used by Trout anglers (Patil et al., 2015), the population is also highly inbred.

Fig 1.1 Google image of the interconnected Lakes Sorell and Crescent showing their location (inset) in the central highlands of Tasmania, Australia.

1.1.3 Carp in research

In laboratory experiments, individuals with unknown genetic background and domestication history impose greater uncertainty, making it difficult to reproduce

experimental results. Since carp has a long history of domestication, it is an ideal candidate for relevant laboratory experiments (Bongers et al., 1998). For example, various strains of carp have been developed to investigate the induction of homozygosity, genetic markers, and the effect of inbreeding and oncogenicity (for review see Bongers et al., 1998). Similarly, to understand the breeding value and to preserve the original gene pool, a live gene bank of common carp strains has been developed (Gorda et al., 1995).

The wide availability and economic importance of common carp make this species an excellent subject for gene and genome manipulation studies, such as the production of androgenic (Bongers et al., 1999; Bongers et al., 1994) and gynogenetic individuals (Horvath and Orban, 1995), ploidy manipulation e.g. production of triploids (Cherfas et al., 1990; Cherfas et al., 1995; Gervai et al., 1980) and tetraploids (Linhart et al., 1991; Recoubratsky et al., 1989), cloning (Yan et al., 1984), and development of transgenic lines (Chen et al., 1993; Sun et al., 2005; Wu et al., 2003; Zhang et al., 1990).

Although carp has been established as an important research species, several aspects of its genome are relatively (compared to human and zebrafish) less characterised. This is partly due to the evolutionary allotetraploidization of carp (Chistiakov and Voronova, 2009; Zhang et al., 2008) that has resulted in a complex genome with twice as many chromosomes (2n=100) compared to most other cyprinids (Chistiakov and Voronova, 2009; David et al., 2003). Additionally, carp contain many genes that are still expressed in duplicates (Barney et al., 2008; Engel et al., 1971), which increases the complexity of characterising and isolating their genes. However, with the increasing importance of carp, either as an aquaculture or a pest species, more studies are exploring the genomic features of carp, making them an excellent target species for research. For instance, a draft genome of carp has been published (Xu et al., 2014), providing a point of reference for diverse investigations. Furthermore, carp-specific molecular information in the public protein

and nucleotide databases has been rapidly increasing over the last decade. For example, 187780 nucleotide and 119377 protein sequences are available for *Cyprinus carpio* in the NCBI database as of Oct 2018, compared to only 2262 and 2325 available, respectively, in July 2009 (Barney, 2010).

1.1.4 Carp control and eradication

Approaches to control and eradicate carp can be distinguished into three categories: physical, biological and chemical. Physically removing carp through different fishing methods (IFS, 2011) is the most widely used technique. The use of pheromones (Adair, 2015), food or metabolites (Haynes, 2011) have also be used to augment the physical removal of carp by designated traps. Biological controls include the introduction of biological agents that can selectively kill carp e.g. Spring Viraemia of carp virus (Crane and Eaton, 1996), koi herpesvirus (Hedrick et al., 2000) or by manipulating sex ratios through the introduction of daughterless traits (Haynes, 2011; Thresher and Bax, 2003). Chemical controls include the use of chemicals like Rotenone, Antimycin and Acrolein (Sanger and Koehn, 1996).

However, complete removal of carp using one particular method is particularly difficult as the mobility and high fecundity of carp allows this species to repopulate quickly. Once established in a particular water body, carp can reach very high densities in a very short period (Gilligan, 2005; Singh et al., 2010; Singh et al., 2013). Additionally, each of the methods has its own limitations. For example, complete physical removal of carp is ineffective on a large spatial scale (e.g. open river systems) as they only remove a part of the population with each attempt. Similarly, biological agents such as viruses could mutate and affect native species (Crane and Eaton, 1996). While many of the chemicals are relatively safe to use considering their low mammalian toxicity, they are expensive for large-scale operations (Sanger and Koehn, 1996) and not applicable for a population

sharing habitat with native aquatic species (Diggle et al., 2012). Therefore, there is urgent need to develop new control methods that are highly effective and not adverse to native biodiversity.

1.2 Gonadal abnormality in fish

Teleost fish are the largest and most diverse class of vertebrates (Volff, 2005). Their abundance, close association with environment and diversity make them an ideal subject for any biological investigations including the reproductive system. Investigation of reproductive abnormalities are considered as a way of understanding the reproductive biology. While the incidence of gonadal abnormality can differ based on the geographic locations, environment and species, some general form of gonadal abnormalities are listed below-

1.2.1 Gonadal abnormality due to the changes of structure and function

Reproductive failures as a result of abnormalities in the size, shape or function of fish gonads are considered rare (Groff, 2004; Gupta and Meske, 1976; Hawkins et al., 1996). Despite this, structural abnormalities of gonads have been found in common carp *Cyprinus carpio* (Gupta and Meske, 1976) and trout *Salvelinus namaycush* (Fitzsimons and Cairns, 2000). In contrast, altered gonads are frequent than those with structural abnormality for many fish species, including trout *Salvelinus namaycush* (Fitzsimons and Cairns, 2000; Ruby and Cairns, 1983), roach *Rutilus rutilus* (Jobling et al., 1998; Wiklund, 1996; Wiklund and Bylund, 1994), whitefish *Coregonus* sp. (Bernet et al., 2004), pacific salmon *Oncorhynchus* spp. (Kinnison et al., 2000), mullet *Mugil cephalus* (Ferreira et al., 2004) and carp *Cyprinus carpio* (Solé et al., 2003). Meanwhile, the reason for structural abnormality remained mostly unknown to date (Bittner et al., 2011).

1.2.2 Gonadal abnormality due to Neoplasm

Neoplasm, defined as any new and abnormal tissue growth in parts of the body, may share some similar characteristics to cancer. Neoplasms can develop on any tissue except adult neurons, which are not capable of division. Neoplasms are most common in skin, gills, liver, and gut, where the cell proliferation is more active and more likely to be exposed to noxious influences like toxicants and pollutants (Roberts, 2012). As highly prolific tissue, gonads are especially susceptible to the formation of neoplasms. Gonadal neoplasms can be divided into two broad categories according to their origin: 1) Germ-cell and 2) Non-germ cell. Cases of spontaneous germ cell tumours/neoplasms have been reported in several species of fish, including barbel *Barbus barbus* (Palikova et al., 2007); zebra fish *Danio rerio* (Basten et al., 2013a; Kent et al., 2007; Smolowitz et al., 2002); African lungfishes *Protopterus aethiopicus* (Masahito et al., 1984) and *Protopterus dolloi* (Hubbard and Fletcher, 1985); Japanese medaka *Oryzias latipes* (Hawkins et al., 1996); black seabass *Centropristis striata* L. (Weisse et al., 2002); bagrid catfish *Hemibagrus macropterus* (Majeed and Wang, 1994); yellow perch *Perca flavescens* (Blazer, 2002) and mixed germ cell-stromal testicular neoplasia in a crucian carp *Carassius carassius* (Fregeneda-Grandes et al., 2010). While non-germ cell originated neoplasms are rare, isolated occurrences have been reported in wild blue shark *Prionace glauca* (Borucinska et al., 2003), leiomyoma in large-mouth bass *Micropterus salmoides* (Herman and Landolt, 1975) and yellow perch *Perca flavescens* (Budd et al., 1975).

Many factors including age, sex, viral infection, environmental pollutants, physical agents, hormone imbalance, genetic, immunological and physiological makeup of the individual (Roberts, 2012) are suspected to trigger neoplasms. Although many factors are attributed to the initiation and development of neoplasms, the genetics behind the formation of neoplasm-related abnormality remains poorly understood.

1.2.3 Gonadal abnormality due to environmental pollution

Gonad abnormalities are also known to occur when individuals are exposed to sewage or wastewater treatment effluents (Dickman and Steele, 1986; Harries et al., 1997; Jobling et al., 1998; Petrovic et al., 2002; Solé et al., 2003; Woodling et al., 2006). Broadly, a diverse group of endocrine-disrupting chemicals (natural or synthetic) present in the environment can elicit an estrogenic response that interferes with normal endocrine and reproductive function (Arcand-Hoy and Benson, 1998).

1.3 Carp as an important species to study gonadal abnormalities

Certain species are well known for their tendency to develop cancer and related gonadal abnormality due to the genetic predisposition. This occurs primarily due to differences in habitat, modes of feeding, contact with sediments and their metabolism including variation in DNA repair capability (Grizzle and Goodwin, 2010). Relatively frequent occurrence and, diversity of gonadal abnormalities make carp an ideal system for their studies. Although gonadal abnormality has been encountered in other species of fish such as shark and rays (Grizzle and Goodwin, 2010), relatively few reports are available as the captivity of those animals is extremely difficult. Similarly, many mutant lines of zebrafish develop spontaneous neoplasm (Amsterdam et al., 2004; Berghmans et al., 2005a; Berghmans et al., 2005b) but gonad-specific abnormalities are rare. Of the available models for cancer, Zebrafish only serve as a model for seminoma (Basten et al., 2013b), indicating a need to develop models for other subtypes of gonadal abnormality. As is documented in the literature, carp are known to exhibit many naturally occurring gonadal abnormalities. These abnormalities can be distinguished into three broad categories as outlined in 1.3.1, 1.3.2 and 1.3.3 below.

1.3.1 Neoplasm and related abnormality in ornamental koi

Tumour and neoplasm related abnormalities in ornamental carp (Nishikigoi) is well known from Japanese breeders, as well as from various historical documents. However, systematic documentation of these abnormality was not well documented until Ishikawa (1977) established the first systematic study of gonadal abnormalities in carp, with affected fish exhibiting a range of associated symptoms/traits such as impaired motility, rapid growth and mortality. Histological and ultrastructural observations indicated those to be dysgerminoma, seminoma (Sirri et al., 2010), theca cell tumours, granulosa cell tumours or both theca-granulosa cell tumours (Ishikawa and Takayama, 1977; Ishikawa et al., 1976). Since ornamental koi are a variant (sub type) of *Cyprinus carpio* developed through repeated artificial mating, the high degree of inbreeding has probably contributed to the gonadal abnormality of ornamental carp. To date, neoplasms in ornamental carp have served as a significant model for understanding neoplasms of aquatic animals in general (Groff, 2004; Ishikawa and Takayama, 1977).

1.3.2 Neoplasm and related abnormality in carp hybrids

The common carp are known to naturally hybridise with closely related species of carp, most commonly with gold fish and crucian carp. Experimental hybridisation with more distant species such as Indian Major carps has also been attempted (Hulata, 1995). The hybrids are generally sterile, but often occurrences of gonadal abnormality are common in the hybrids (Dickman and Steele, 1986; Down and Leatherland, 1989; Granado-Lorencio et al., 1987; Leatherland and Sonstegard, 1978; Leatherland and Down, 2001; Sonstegard, 1977). While the frequency of gonadal abnormality can differ in different age classes, up to 100% of older male hybrids with goldfish were found exhibiting gonadal abnormality (Down and Leatherland, 1989). The mechanism of the abnormality was not clear, however observed hyperplasia of the pituitary gland leading to the overproduction of testosterone (T), 11-Ketotestosterone (11-KT) and estradiol-17ß (E2)

has been hypothesized for this abnormality (Down et al., 1988). Nevertheless, the connection of hormonal imbalances on oncogenesis remains unclear (Down, 1984). It is also possible that hybridization between two different species can result in incompatibility between sex determining genes or even the somatic chromosome, making them vulnerable to neoplasia (Down and Leatherland, 1989).

1.3.3 Neoplasm in carp

Reports of gonadal abnormality in the parental strain of the carp is much less common than in ornamental carp (i.e. Nishikigoi) or in hybrids (i.e. goldfish or crucian carp). However, few cases have reported abnormalities associated with pollution from heavy industrialization activity (Hassanin et al., 2002; Petrovic et al., 2002; Solé et al., 2003). All incidents were attributed to the endocrine disruptive chemical present in the habitat

Table1: Gonadal abnormality in carp and their probable etiology-

Variant/ Hybrids	Origin/symptom	Diagnosis	Number of fish affected	Sex	Attributed reason	References
Nishikigoi (Ornamental carp)	Germ cells and ovarian mesenchyme	Dysgerminoma, Granulosa cell, Thecal cell, embryonal carcinoma, teratoma	>100	F	Genetic	(Ishikawa and Takayama, 1977; Ishikawa et al., 1976)
Shiro bekko koi carp (Ornamental carp)	Epithelial originated gonadal neoplasm	Undifferentiated ovarian carcinoma	One	F	Unknown	(Raidal et al., 2006)
Koi carp (Ornamental carp)	Undifferentiated Seminal cells	Seminoma	One	M	Unknown	(Sirri et al., 2010)
Koi carp (Ornamental carp)	Mesenchymal neoplasm	unknown	>100	Unknown	Unknown	(Knuesel et al., 2015)
Hybrids with goldfish (Carassius auratus)	Sertoli, Germ, Stromal cells	Gonadal neoplasm	>100	M	Genetic	(Dickman and Steele, 1986; Leatherland and Sonstegard, 1978)
Hybrids with Goldfish Carassius auratus	Sertoli, Germ, Stromal cell, Seminomas	Gonadal neoplasm	>100	Both	Genetic	(Down, 1984; Down and Leatherland, 1989)
Hybrid with Carassius auratus	Gonad cells	Seminoma	One	M	Unknown	(Chiba et al., 1979)
Hybrid with Crucian carp (Carassius carassius)	Germ cells and Sertoli cells	Dysgerminoma, seminoma, leiomyomas	>100	M	Genetic	(Granado-Lorencio et al., 1987)
Hybrids between Chinese and European strains	Reduced or missing gonad, few eggs in female gonad	Unknown	13	Both	Partial genetic barrier	(Hulata et al., 1980)

Carp	Hermaphroditism, unequal gonad	Unknown	Some	More in Male	Unknown	(Gupta and Meske, 1976)
Carp	Reduced GSI, failure of milting, Oviduct in male	Ovo-testis, Functional abnormality	Unknown	M	Toxicants	(Gimeno et al., 1998a; Gimeno et al., 1998b)
Carp	Reduced GSI, Arrested spermatogenesis	Testicular atrophy, intersexuality	Unknown	Both	Pollution	(Hassanin et al., 2002; Jenkins and Goodbred, 2005; Lavado et al., 2004; Sole et al., 2003)

1.4 A unique gonadal abnormality in Tasmanian carp

As part of an ongoing integrated carp management program, the Inland Fisheries Service Tasmania (IFS) encountered a condition where the gonads of carp appear like lumps of jelly (0.5 to 2 cm in diameter) and affected fish are "sterile" (i.e. incapable of discharging gametes). Although the affected fish do not show any apparent external morphological deformities, apart from expelling watery liquid instead of milt, the condition of the gonad itself exhibits gross morphological diversity with no apparent signs of neoplasm or pathogenic infection. It appears that this sterile condition, henceforth referred to as jelly-like gonad condition (JGC), has not been previously encountered or reported elsewhere for carp. Briefly, JGC in Tasmanian carp appears unique compared to previously reported gonadal abnormalities in fish or carp. Being situated at the head of the River Clyde, Lake Sorell is less vulnerable to environmental pollution. Additionally, the absence of goldfish and other carp species rules out the possibility of hybridization. No mortality has been observed due to JGC. However, as Lake Sorell carp population originated from a handful of fish escaped from live bait used by the anglers (Patil et al., 2015), the degree of inbreeding would probably be extremely high resulting a genetic predisposition that is primarily responsible for the observed mass gonadal abnormality.

This study aims to explore its prevalence, severity, histopathology, growth, fertility, relation to biotic and abiotic factor and its potential genetic drivers. In order to investigate the molecular process triggering JGC in Tasmanian carp, this study took advantage of state of the art next generation transcriptome sequencing. The application of high throughput next generation sequencing technologies to understand the transcriptomic expression has profoundly changed our way of understanding the biology of rare conditions (Reuter et al., 2015). RNA-seq technology relies on sequencing, mapping and then quantifying the total transcribed RNA (Wang et al., 2009). It has several advantages over the conventional micro-array and other previously described methods. For instance,

it allows for the entire transcriptome to be examined instead of being limited to known sets of candidate genes. Furthermore, it facilitates the detection of isoforms, novel transcriptome, non-coding genes, allelic variation, mutations in the coding region and differential expression (Chen et al., 2011; Wang et al., 2009; Zhao et al., 2014). Importantly, RNAseq does not suffer from background noise associated with techniques such as microarray, which often provides false positive results (Pinto et al., 2011). However, RNAseq data analysis is more complex than with traditional microarray, as it generates a massive quantity of data that requires a combination of advanced bioinformatic and statistical tools for analyses (Chen et al., 2011).

This study describes and explores potential causes of a unique gonadal abnormality in an inbred strain of carp.

1.5 Objectives

The overarching objective of the study was to characterise a naturally occurring and unique jelly-like gonad condition in the carp and elucidate its causes.

Specifically, this thesis will:

1) Determine the characteristics of JGC, including prevalence, morphology and histopathology;
2) Test relatedness of the condition to select biotic and abiotic factors and;
3) Discern potential genetic cause and response in its development.

1.6 Thesis structure

Thesis chapters in this study presents original data written as manuscript formats for submission to peer reviewed journals. Each experimental chapter contains separate

introduction, materials and methods, results and discussion, with a single and consolidated reference section at the end. A summary of the thesis structure is provided below-

Chapter 1: General Introduction

An introduction to the Common carp, highlighting the background associated with the research topic and objective of the investigation.

Chapter 2:

Contains a general characterization of the JGC condition including its epidemiology, gross morphology, severity stages, histology, progression and an evaluation of the condition as a non-germ cell cancer.

Chapter 3:

Presents the relatedness of JGC to select biotic and abiotic variables. Growth, fertility and the endocrine response of the JGC fish were also investigated to evaluate the potential for JGC carp for aquaculture and management of pest populations.

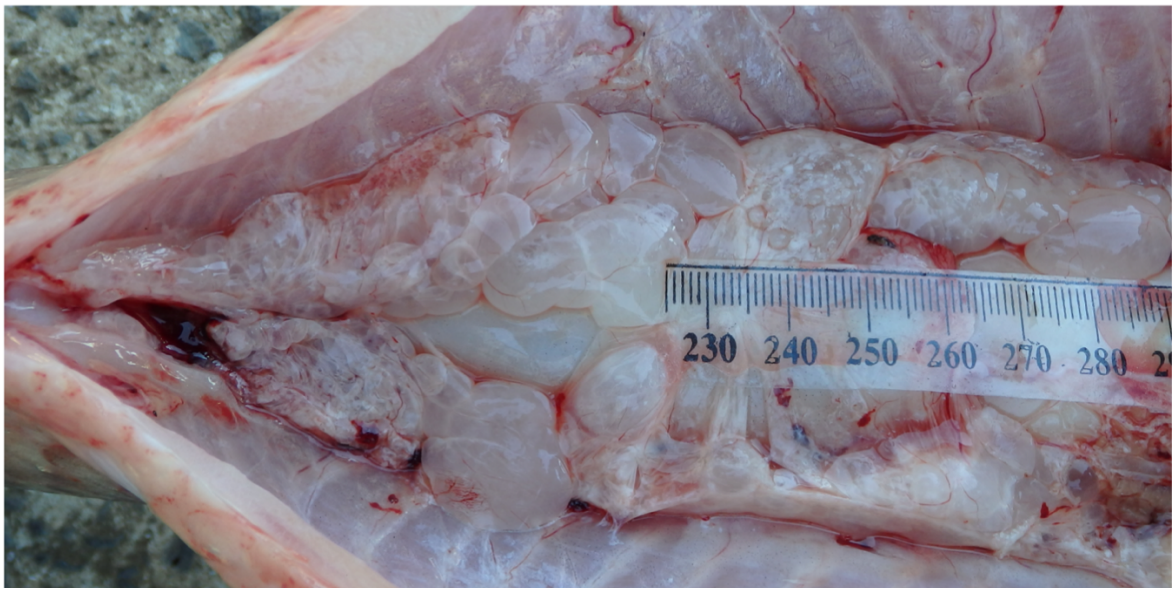
Chapter 4:

Presents RNAseq transcriptome analyses of JGC and normal carp, followed by identification of biological pathways associated with the condition.

Chapter 5:

Provides a review of the overall findings with a general discussion of broader applications, highlighting key findings with future recommendations.

Chapter 2: A novel testicular abnormality in an inbred wild population of the common carp *Cyprinus carpio* (L).



2.1 Abstract

Gonad abnormalities can restrict or completely block reproductive capability of individuals and in some case that of their populations. Here we describe a novel cancer-like testicular degenerative condition of non-germ cell origin with a high prevalence (up to 22.1% of the population) in a wild population of carp. Based on gross morphology the condition shows progressive severity which could be categorised into low, mild, severe and complete. In early stages of the condition, an abnormally increased proliferation (11 fold) of the Sertoli cell was observed, followed by apoptosis of all testicular cells resulting in fluid filled blisters in the later stages. This initial uncontrolled proliferation of Sertoli cells suggests that the condition is likely triggered by malignant pathways. Although few cases of Sertoli cell malignancy have been reported previously, the observed subsequent apoptosis of all testicular cells *en masse*, rendering the animals 'sterile' appears unique. Observations to date, indicate that this condition is specific to male carp and not present in other species of fish sharing the habitat. High prevalence of the condition allowed comparative evaluation between affected individuals, an aspect likely to facilitate future studies, including robust testing of therapies. Particularly, where there is a knowledge gap on Sertoli cell malignancy and testicular cell apoptosis, this cohort of fish could serve as a valuable system.

2.2 Introduction:

Although less common, degeneration of testicular tissue is a pathological condition that results in disruption of the natural architecture of seminiferous tubules and testicular epithelium with a consequent loss of testicular function. The causes for the condition may vary from physical stress (e.g. exposure of heat, cold or radiation) (Aksglaede et al., 2005; Blanchard et al., 1996; Turner, 2007) to diseases (e.g. cancer) (Wood and Elder, 2009), but the mechanisms remain poorly understood. The etiology and pathogenesis of

non-cancerous testicular degeneration is very limited as only a few naturally occurring reports are available (Blanchard et al., 1996; Turner, 2007), however, degenerative changes due to cancer and neoplasm are far more common. This occurs primarily due to the adverse effect of toxicants produced a result of extreme cell division in cancer tissue (for review see Ames et al., 1993).While, testicular degeneration due to exposures of physical stress can be avoided easily, intrinsic factors particularly those caused by cancer and neoplasm are extremely difficult to manage.

Like most cancers, testicular cancers present grave consequences and occur frequently with no definitive cause. Moreover, the occurrence of testicular cancer in humans has been increasing at an annual rate of 2.3-5.2% in some industrialised countries (Adami et al., 1994). There is some evidence to suggest that incidences of testicular cancer are demographically linked. For example, white and black males exhibit similar histopathological appearances and age distribution (Moul et al., 1994) but displayed different progression rates i.e. increased at a rate of 3-6% annually for white males (Oosterhuis and Looijenga, 2005), but remained unchanged at ~5% for black males (Moul et al., 1994; Van Den Eeden and Weiss, 1989).

Tumours and cancers comprise a heterogeneous collection of cells with varying proliferation capability often resulting in a more abnormal structure than their original organisation (Al-Hajj and Clarke, 2004; Heppner, 1984). Testicular cancer can originate from any cell type within the testicle and can be grouped into two broad categories based on their origin – germ cell and non-germ cell (Eble, 2004). Tumours derived from germ cell origin constitute 90-95% of total testicular cancers in humans (Dilworth et al., 1991), affecting 1 in every 500 Caucasian males (Basten et al., 2013b). Recent clinical classification has distinguished five different subgroups of the testicular germ cell cancer (TGCC) based on chromosomal complements and development (Oosterhuis and

Looijenga, 2005). Several studies have been conducted to understand the cause of TGCC. For example, involvement of aneuploidy and chromosomal changes such as gaining short arm by chromosome 12 to seminoma and non-seminoma (Sandberg et al., 1996); gaining portions on chromosome 9 with sprematocytic seminoma (Rosenberg et al., 1998); loss of 1p, 4 and 6q, and gain of 1q, 12 and 20q region to type I yolk sac tumours (Oosterhuis and Looijenga, 2005) have been implied. The possible roles of tumour suppressor genes have also been investigated and reviewed (Looijenga and Oosterhuis, 2002; von Eyben, 2004). Testicular germ cell tumours (TGCTs) have been extensively studied using animal systems as these offer ready insights into understanding the causes and assist in developing suitable therapies. As a result, non-human vertebrate models are now available for almost all subtypes of germ cell testicular cancers (Basten et al., 2013b; Neumann et al., 2011; Oosterhuis and Looijenga, 2005). However, non-germ cell tumours/cancers that originate from Leydig cells, Sertoli cells, theca cells, granulosa cells or mixed cell types (Dilworth et al., 1991) remain poorly investigated. This is also complicated by lack of clarity as to what constitutes a non-germ cell tumour. For example, generic testicular lesions and secondary tumour-like conditions are all grouped as non-germ cell tumours (Dilworth et al., 1991).

Uniquely, a male-specific testicular degenerative condition in a wild parental (non-ornamental variants) population of carp (*Cyprinus carpio*) was encountered. Here, the gonads (testis) of the carp appear as lumps of jelly, the affected fish are ‘sterile’ but otherwise appear healthy. Preliminary histology investigations suggested multiple symptoms, ranging from proliferation of somatic cells at the expense of germ cells. The surprisingly high incidence of testicular abnormality in carp in this isolated and pristine highland lake with no reported incident of pollution is intriguing. Nonetheless, the high prevalence of the testicular abnormality has allowed an opportunity to scrutinise the onset and progression of this condition/disease. As a first step towards understanding the

aetiology of the condition and potentially establishing an animal model that mimics an as yet unidentified human condition of male sterility, this study systematically investigated the prevalence, histopathology and its developmental progression. Morphologically, the affected gonads appear 'jelly-like' and hence designated as 'Jelly-like gonad condition" (JGC).

2.3 Materials and methods

2.3.1 Sample collection

The fish used in the study were captured as part of an integrated carp management program by Inland Fisheries Service (IFS), Tasmania at Lake Sorell (42°06' S, 147°10' E). While carp were the target species of eradication, by-catch of other fish species inhabiting the lake namely the golden galaxid (*Galaxias auratus*), brown trout (*Salmo trutta*) and long-finned eel (*Anguilla australis*) were also made available for the study.

Fish caught over six fishing/spawning seasons (October-March of each year) between 2011 to 2017, were examined. Interestingly, all fish analysed originated from a recruitment event in 2009 and hence represent a single year class or cohort. The gonads of every carp caught from the lake between 2011 and 2017, with the exception of decomposed carcasses were checked thoroughly and samples from a subsample of affected and unaffected fish were collected for morphological and histological analyses. To minimise potential seasonal effects, histological samples were collected in December 2015.

2.3.2 Morphological observation

Gross morphological analysis was performed on digital images of gonads using ImageJ. Gonad length, visible surface area and affected area were marked and measured. Blister size was calculated by measuring 10 random blisters from each sample. Distinguishable

patterns of abnormality were further subdivided into a severity category to establish disease progression. All the statistical analyses were performed using R.

2.3.3 Histopathology

For histological examination, JGC and control tissue were fixed using standard protocols and embedded in paraffin wax overnight. Three histological stains namely, haematoxylin and eosin (H & E), Mallory's triple stain and PAS alcian blue were used. Sections fixed in formalin were later post-fixed in Bouin's fixative for 12h to obtain the effect of picric acid base fixatives, when required. Mallory's stain was used to visualise collagen, distinguish the dead and live cells and haemorrhages from blood cells; whereas the PAS alcian blue was used to find pas positive cells (especially melano-macrophage aggregation). To better understand cellular changes, quantitative cell count was performed in histological subsamples from each severity stage (n=3, H&E sections). Randomly selected non overlapping fields (n=3) of 0.115 mm^2 (at 400x) from each of the sample were marked and cell count was performed using NIS image analysis software.

2.3.4 TUNEL assay

TUNEL assay was used to detect fragmented DNA of apoptotic cells in wax embedded JGC and control tissue. Fluorescent images were visualised using Leica DM LB2 microscope (excitation: 450-500 nm and emission: 515-565 nm).

2.4 Results:

2.4.1 Gross appearance of the affected gonads

Of all the 6111 carp examined (between 2011-2017) the JGC condition was observed in 18.51% of male fish (487 of a total of 2631 male fish). Healthy carp testes were found as white, well-structured cylindrical organ (Figure 2.1b) while JGC tissue contained blisters

of various size and consistency. In the early stages of JGC, small blisters appeared on the gonadal surface with some association of structural abnormality unlike the cylindrical structure of normal carp testis. In an advanced stage, multi-nodular fluid-filled clear or semi opaque blisters were found over and inside of the gonad. Completely affected tissue appeared spongy eventually turning to fluid filled vesicles. In early stages, the blisters were filled with slightly viscous fluid, but in advanced stages these were mostly filled with clear liquid, often with a consistency comparable to water. However, invasion of other organs was never seen in any samples (n=487) examined. The initiation site of JGC had no correlation/preference with gonad topology as it was found to occur all over the gonad (anterior, posterior or mid region), albeit varying between individuals. Completely affected tissue appeared as 'a bunch of grapes'(Figure 2.1a).

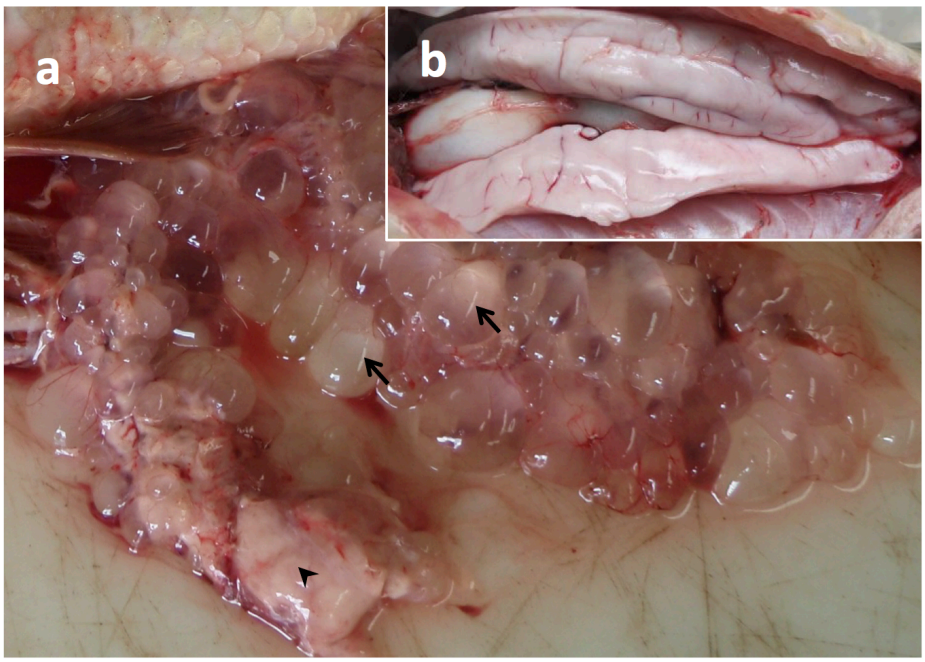


Fig 2.1 a) Gross appearance of an advanced JGC testis. In advanced stages, the entire testis is covered with jelly like blisters (arrows) giving a 'bunch of grape' like appearances. Unaffected tissue can also be seen (arrow head) b) healthy carp testes.

2.4.2 Prevalence of the disease

Investigation of all fish (n=6111) caught over six different seasons (season 11/12- 16/17) revealed that the proportion of JGC increased over subsequent seasons/years (Figure 2.2). In the 2011/12 season most of fish were immature (90.23%) and JGC was not recorded. In 2013/14 the proportion of JGC sharply increased to 8.6% from 0.3% in season 12/13. Similarly, when the proportion of JGC was calculated as a percentage of male fish, a rise in the percentage was observed from 15.2% (in 13/14) to 17.30% (in 14/15). In summary, steady increases in the proportion of JGC fish in Lake Sorell were observed over time reaching a proportion of 45.99% of males (22.11% of the total fish caught) in the 16-17 season (Figure 2.2).

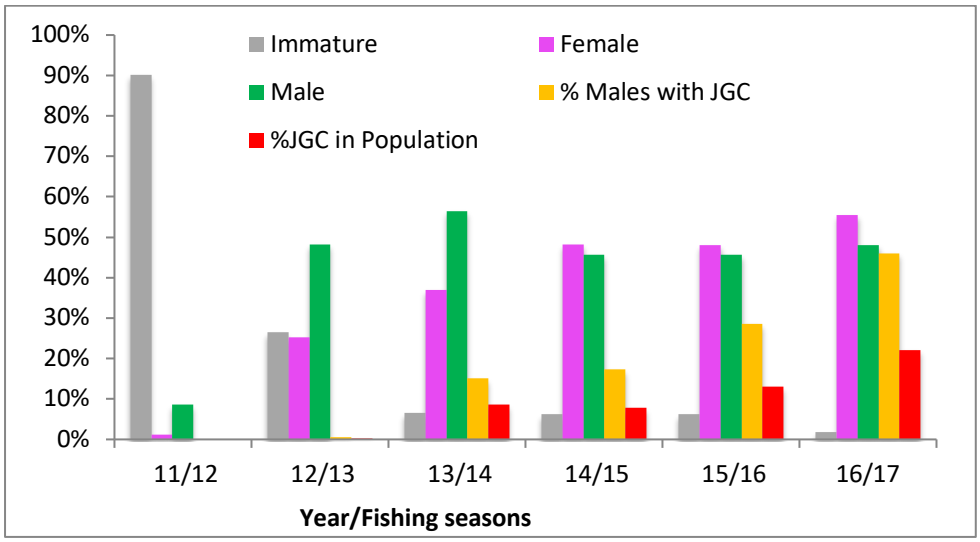


Fig 2.2 Prevalence of JGC observed over six seasons/years.

2.4.3 Morphological analysis of the disease

Of the 56 JGC affected fish analysed from two consecutive years (2015, 37 individuals; 2016, 19 individuals), all but one fish had both gonads affected. Based on the extent of affected tissue and blister size the severity of the condition could be categorised into four sub-categories:

1) Low (Stage 1): Characterised by small (between 5-20 mm^2, average 14.5 ± 3.9 mm^2), localised blisters (n < 20) with a generally well-structured gonad but with up to 1-33.33% of the gonad affected.

2) Mild (Stage 2): Blisters generally larger in size (average 25.1 ± 3.0 mm^2), and number (21-99) on the gonad but fair amount of good tissue is still visible. The affected area constituted between 33.34-66.67% of the testis.

3) Severe (Stage 3): Large (average 44.5 ± 4.2 mm^2) and numerous (100-500), blisters with sparse normal tissue. The affected area covered between 66.68- 99.99% of the testes.

4) Complete (Stage 4): Blisters generally larger in size than the previous stage with average blister size of 52.5 ± 3.8mm^2. More than 500 blisters were visible in each sample with no sign of normal tissue (100% affected).

Gross morphological progression of affected males was monitored over two successive breeding seasons (Figure 2.3). Here, stage 1 JGC rate increased from 16% in 2015 to 31% in 2016 (Figure 2.3a). However, the proportion of mild and complete condition was stable in both years with proportion of the severe condition (stage 3) reducing in 2016. Although weak (coefficient=0.5), a highly significant (p= 9.5e-05) positive correlation was found between percentage of gonad affected and blister size (Figure 2.3b), indicating the size of blister increases with advancing severity. In contrast, a strong (coefficient =0.83) and significant (correlation p=3.5 e-15) relationship existed between left and right gonad lobe affected rates (Figure 2.3c), suggesting both lobes of the testis seemed equally vulnerable.

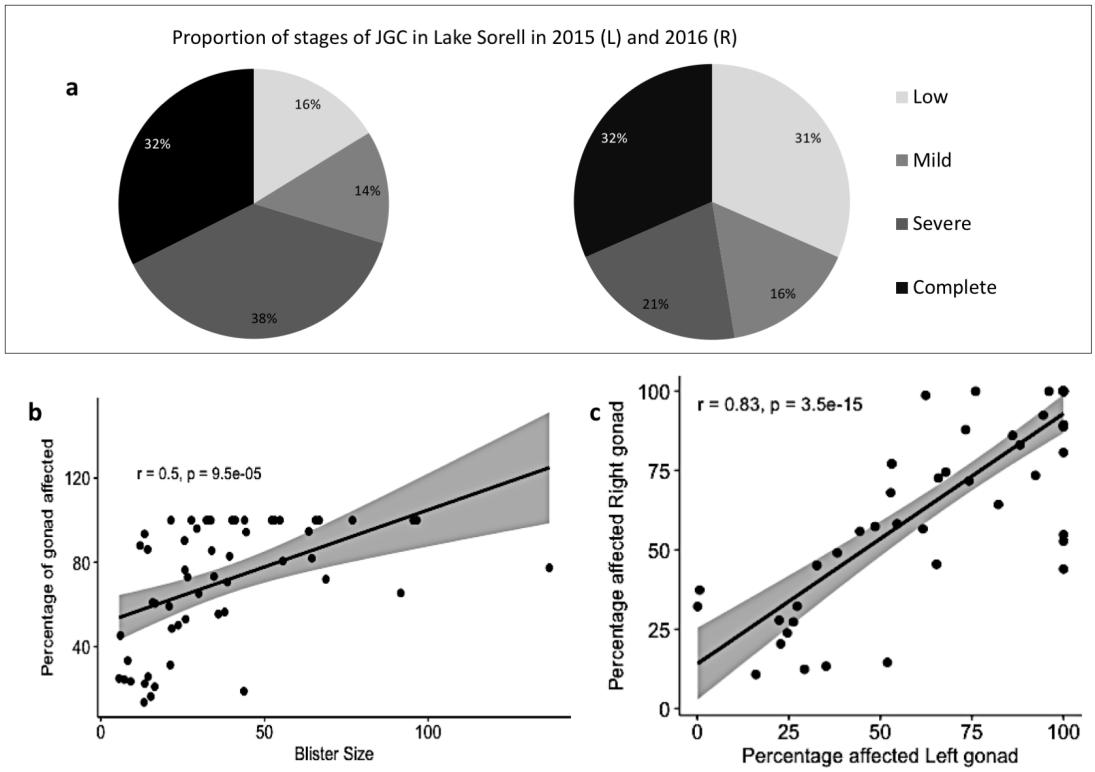


Fig 2.3 Quantitative analyses of JGC a) proportion of the JGC stages in two select years (2015 and 2016 b) relationship of the blister size and percentage tissue affected, c) correlation of left and right gonad affected areas.

2.4.4 Histological observation

2.4.4.1 Histology of Normal testis

Normal testis consisted of healthy seminiferous tubules containing Sertoli cells, epithelial layer and all stages of spermatogenic cells (Figure 2.4). The lamina of the seminiferous tubule was consistently and tightly packed with spermatozoa and a thin layer of interstitial tissue was visible between the seminiferous tubules across all the control samples. Healthy male testes contained nests of maturing germ cells developing synchronously and enclosed by Sertoli cell extensions (Figure 2.4a and b). Adjacent nests were at times separated by junctional complexes and mainly contained different stages of cells. These nests were abundant in the peripheral area of the seminiferous tubules. Sertoli cells (S) were distinguishable by their distinctive cytoplasm, amorphous shape and irregular, lightly stained nucleus (H&E) with a prominent nucleolus (Figure 2.4b). Sertoli cells

were situated around the periphery of the seminiferous tubules in close contact with the basement membrane. No hypertrophy of the Sertoli cells was noted.

Generally, spermatogonia were found either as single type A undifferentiated spermatogonia (Gu, lightly stained invaginated nucleus Figure 2.4b), type A differentiated spermatogonia (Gd), or as a group of secondary Type B spermatogonia (Gb, Figure 2.4b) inside a nest. Nests with fully formed primary spermatocytes (PSt), secondary spermatocyte (SSt) (smaller than SG and more granular and oval outline, Figure 2.4b) and their intermediate stages were also visible. Nests with fully formed spermatid (Sd) were also observed. Mature spermatozoa (Z, Figure 2.4a, b) were always present in the seminiferous lumen free from Sertoli cell connection. Leydig cells (L) and myoid cells were occasionally found in relatively small numbers in the interstitial tissue. Leydig cells were distinguishable by their location, cytoplasmic structure and polyhedral shape. Hypertrophy of the Sertoli cells was not visible in the controls.

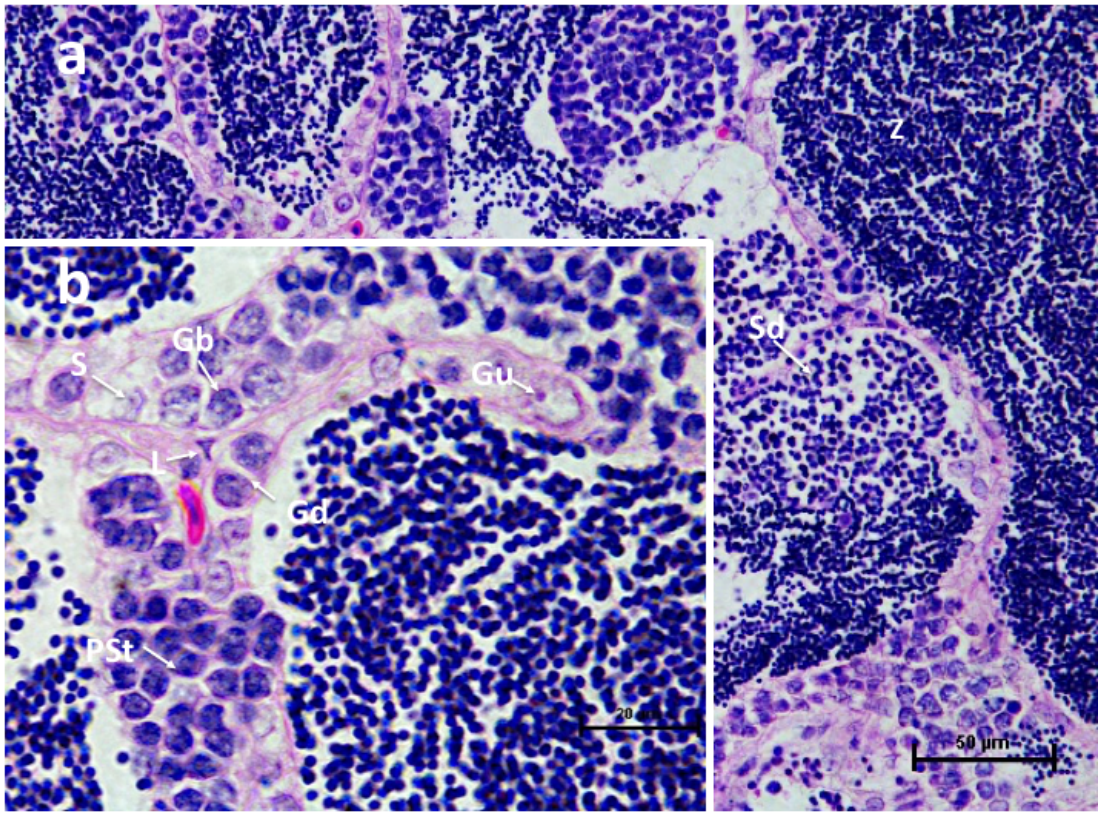


Fig 2.4 Transverse section through a control gonad showing healthy seminiferous tubules containing all stages of spermatogenic cells. S, Sertoli cells; Gu, undifferentiated spermatogonia; Gb, secondary spermatogonia; S, Sertoli cells; L, Leydig cells; Z, spermatozoa; PSt, primary spermatocyte; Sd, spermatid.

2.4.4.2 Histopathology of the Diseased Gonad in progressive stages

The JGC affected gonad displayed a spectrum of testis histo-pathology although several features common to control gonads could be found in all four stages.

2.4.4.2.1 Stage 1

Histopathological examination of representatives of stage 1 JGC revealed a range of changes compared to the control gonad. Generally, the condition appears to originate from a single point (often at the periphery of the testis) spreading inward and radially (Figure 2.5a). The affected area had severely reduced spermatozoa, spermatocytes with complete absence of differentiated spermatids, whereas the morphology of the unaffected portion was similar to the control. The most characteristic feature of the stage 1 JGCs was a marked decrease in the differentiated germ cells (i.e. Spermatids, Spermatozoa).

Progressively, a massive proliferation of structural Sertoli cells was also evident (Figure 2.5c). In most cases, seminiferous tubules became smaller in size (collapsed or absent), due to the proliferation of Sertoli cells (Figure 2.5). Very rarely, Sertoli cell hypertrophy was observed in the sections.

The tunica albuginea of the testis contained a thicker band of connective tissue and more collagenous material between the surface epithelium than the control (Figure 2.5). Leydig cells were difficult to detect in the proliferated mass of cells using only morphology, as the massive proliferation of Sertoli cells created a homogeneous layer, masking most other cell types (Figure 2.5c).

Extravasation or haemorrhage of the RBC (Figure 2.5c, R) was a regular characteristic of JGC tissue. These patchy clumps of RBC were mostly abundant in channels of loosely organised structure and totally absent in control gonad.

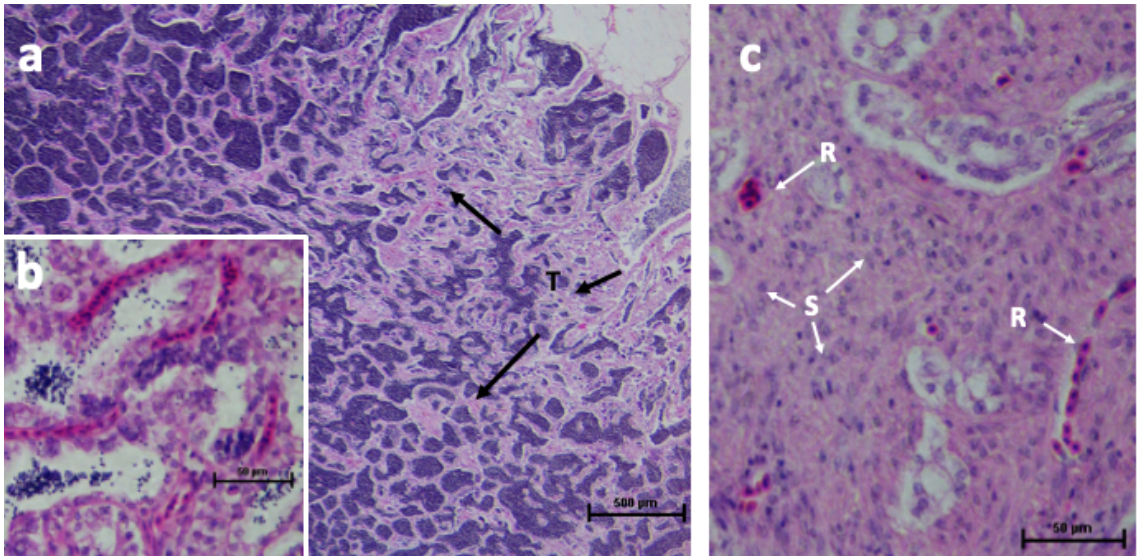


Fig 2.5 Histological section through a representative Stage 1(low) JGC testis. a) Disease progression overview, thick tunica albuginea (T), direction of arrows points to the direction of progression of the condition b) magnified view of a sections with JGC affected area (c) showing proliferation of Sertoli cells (S) and patches of RBC (R).

2.4.4.2.2 Stage 2

Stage 2 was distinguished by further reduction or mostly absence of spermatozoa, which resulted in empty lumen (Figure 2.6a). The remaining spermatogenic nests began to disassemble with testes tissue beginning to disintegrate (Figure 2.6b). Presence of RBC was more prominent in this stage. Spaces within the seminiferous tubules were even smaller with a presence of proliferated mass of the homogeneous cells. Mostly JGC stage 2 testes was firm, collagen infiltrated and non-vascularised mass of tissue with random presence of vacuoles (Figure 2.6a).

Cell death becomes the prominent feature at late stage 2. Karyopyknotic nuclei, membrane blebbing and chromatin condensation were regular features of the dying cells (Figure 2.6d, arrow). However, all blood cells retained their shape and size at this stage (Dark red oval cells, Figure 2.6e Mallory's trichrome). Dead cells were distinguished by no nucleus with lightly stained cytoplasm. In summary, stage 2 was classified by further reduction or even complete absence of spermatozoa and a start of cell death with presence of larger patches of RBC than stage 1.

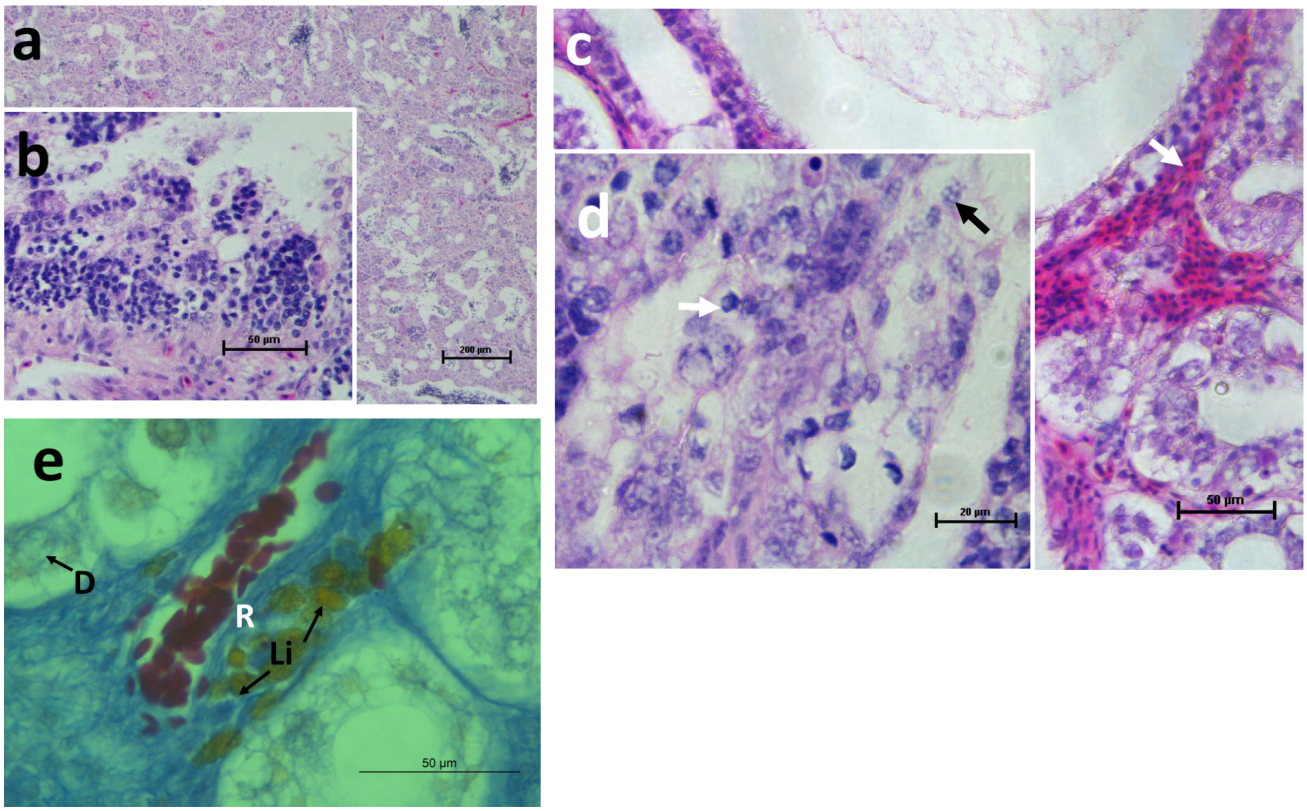


Fig 2.6 Representative histological sections through Stage 2 (mild) JGC testes. a) overview b) with nests of cells disintegrating, c) abundance of RBC due to haemorrhaging and d) signs of apoptosis, Karyopyknotic nuclei (black arrow), chromatin condensation (white arrow). e) Mallory's trichrome staining of the JGC tissue representing the abundance of collagen fibre (stains blue), live cells (Li, stains yellow), dead cells (vacuolated and pale, d), RBC (R, stains red, oval cells in clump).

2.4.4.2.3 Stage 3

Stage 3 was characterised by larger vacuoles than in stage 2 with presence of more fibrous tissue; primarily the remnants of the dead cells (Figure 2.7a), which slowly began to accumulate and degenerate. Spermatozoa, spermatids and spermatocytes were completely absent. The presence of 'live' cells was sparse. Rarely, parts with undifferentiated and differentiated spermatogonia associated with the lumen could be visible and they were much fewer in number than stage 2. Cell death was the most prominent characteristic of this stage. Dead cells were difficult to distinguish by their morphology because of the changes in cellular and nuclear structure or even the size (swelling of cells). Sertoli cells appeared more vacuolated or empty than stage two. Cell

death was noted either as single-celled or in a clump (SG and Sertoli) that appeared detached from the interstitial supporting tissue. Enlargement of vacuoles within the seminiferous tubule was also prominent with two or more empty lumen fusing to form a larger empty space. Conceivably, this space was filled with watery fluid, imparting a spongy appearance as was evident in the gross morphological observations. Interestingly, blood cells were fewer than previous stages with remnant RBC showing signs of disintegration (Figure 2.7b). The pas positive melano-macrophages were prominent. (Figure 2.7c).

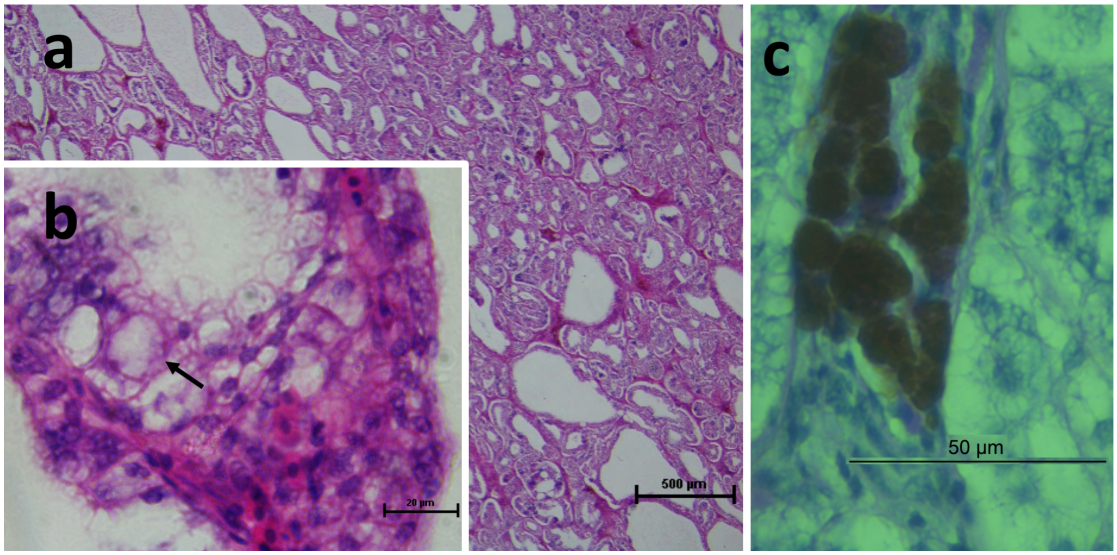


Fig 2.7 a) Overview, histological section of a representative stage 3 JGC showing b) large vacuolated spaces and apoptotic cells with membrane blebbing (arrow). c) Tissue stained with PAS-Alcian blue highlighting the Melano-Macrophage aggregation in interstitial space (darkly stained).

2.4.4.2.4 Stage 4

Stage 4 JGC gonads were characterised by much larger (often > 2.4 mm^2) empty vacuolated spaces bound by a thin layer of connective tissue. Seminiferous tubules were almost impossible to distinguish due to structural disruption. Only those cells found embedded in the interstitial tissue managed to survive at this stage. Sign of cell death was

obvious on the edge of the blister wall. Cell debris in the lumen and RBCs in the interstitial space were almost absent (Figure 2.8).

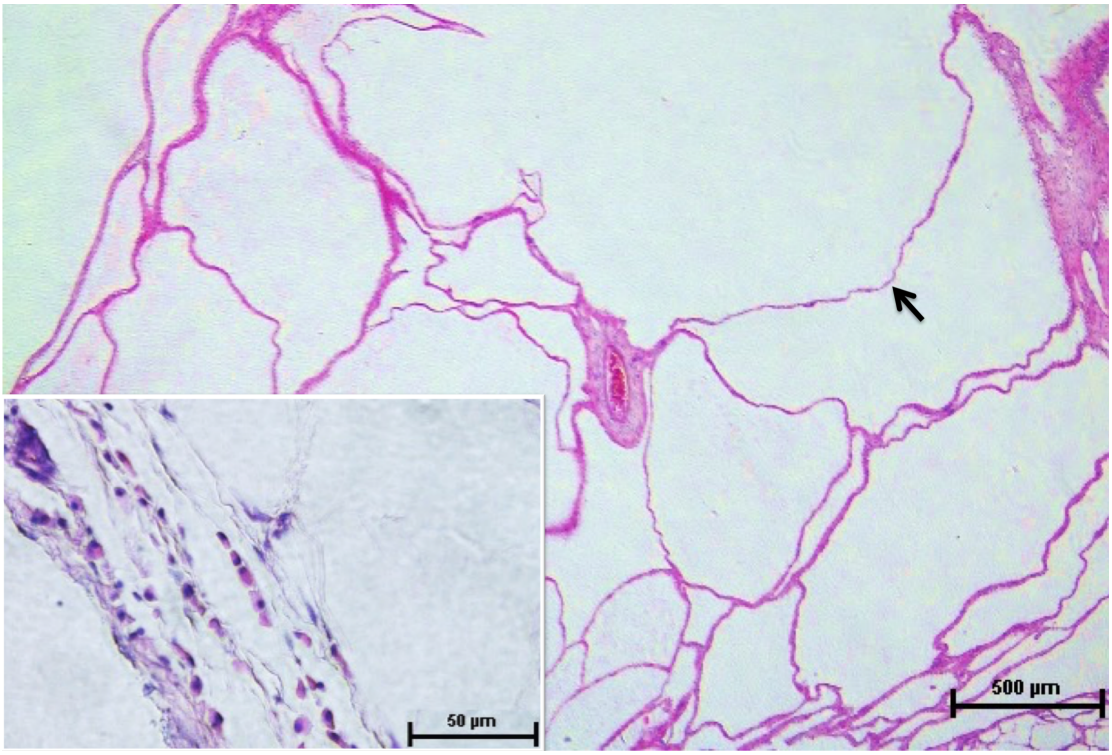


Fig 2.8 Representative histological section through stage 4 JGC. Note, large vacuolar spaces, with little or no sign of cells (see inset for close up) and bound by a thin membrane (arrow).

2.4.5 Quantitative cellular changes

One-way factorial ANOVA of cell types and counts revealed that stage of severity showed a relationship with both cell types (p=2e-16) and their numbers (p=2e-16). The most distinguishable feature of the JGC tissue with advancing severity was a marked reduction of spermatozoa (Figure 2.9). In contrast, the Sertoli cell number was initially greater than in the control (11 folds) in stage 1, but eventually reduced significantly in later stages, but remained higher than control. Although signs of cell death were evident in stage 2 (Mild), it peaked in stage 3.

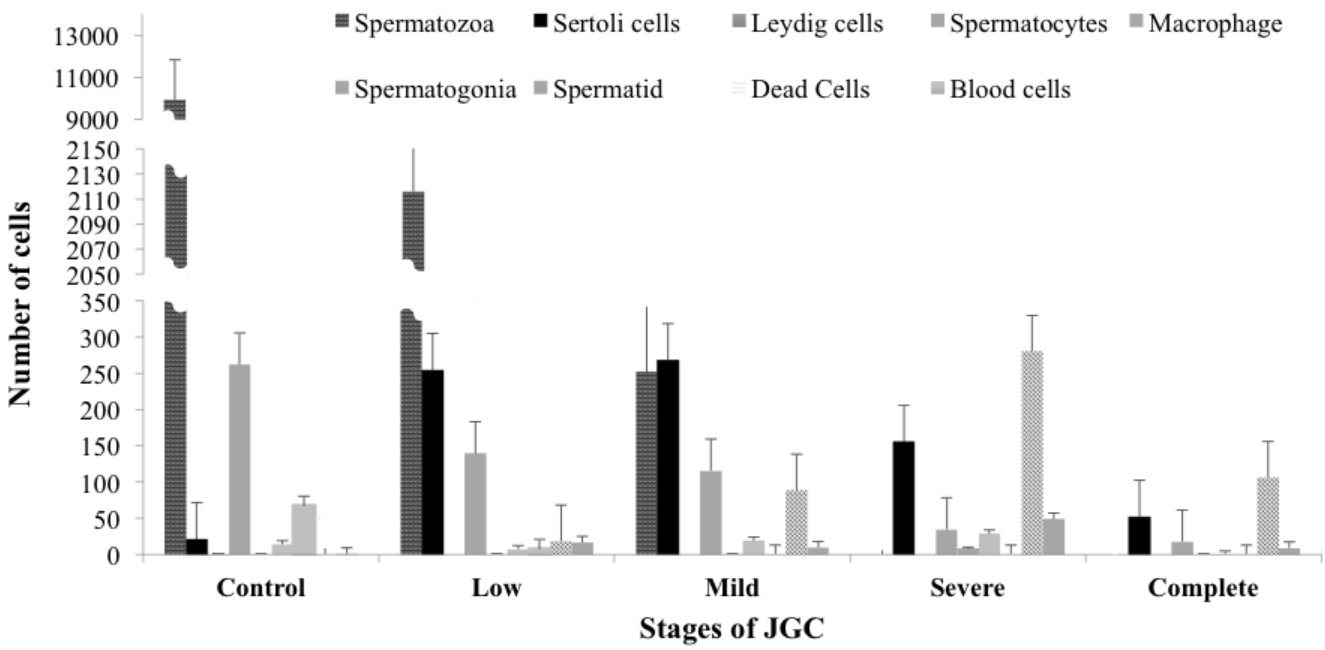


Fig 2.9 Quantitative change of key cell types in control and four stages of JGC tissue. Note abnormally high Sertoli cells in Stage 2 and 3. The count of intermediate germ cells declined significantly with the progression of the condition. (Note: error bars with no crossbar have values in the scale break).

2.4.6 Disease progression

Although, the JGC condition could be categorised into four different severity stages, presence of trace tissues resembling the characters of preceding or following stages could be observed in the same individuals (Figure 2.10). Similarly, in histological sections there was overlapping of tissue morphology between stages. For example, sections through stage 2 individuals contained regions that were also typical of stage 1 and stage 3, albeit sparsely. Similarly, in stage 1 gonads, there were areas which were not affected that had histological characteristics comparable to control gonad.

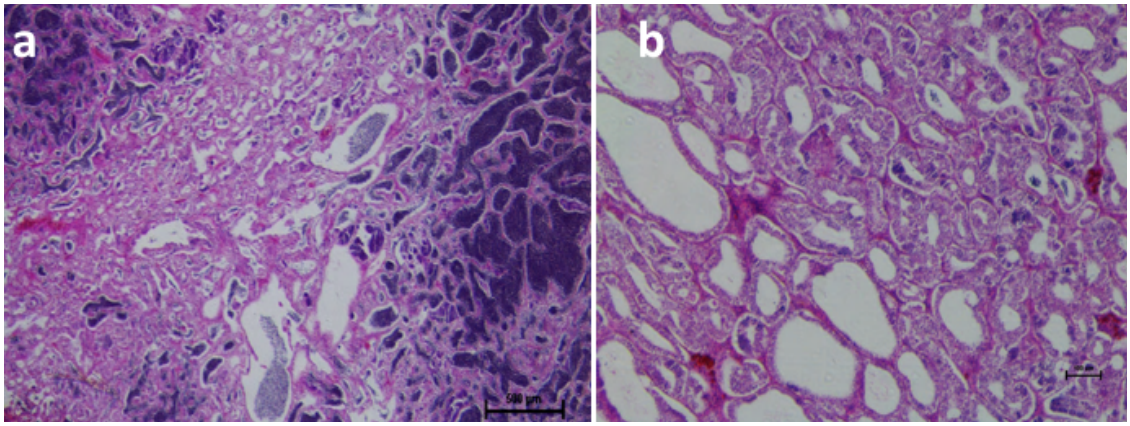


Fig 2.10 Overlapping progression of stages. a) stage 1 sharing features of control tissue in places (darkly stained and compact spermatozoa with more regular organisation of the tissue—right and top left) b) stage 2 section with has some similarity of stage 3 (large vacuolar spaces, bottom left).

2.4.7 TUNEL assay

Sections through control testis did not yield any TUNEL positive signals (Figure 2.11a). In contrast, JGC tissue sections through all stages showed positive signals and the signal intensity increased with advancing stages of severity (Figure 2.11 b-d).

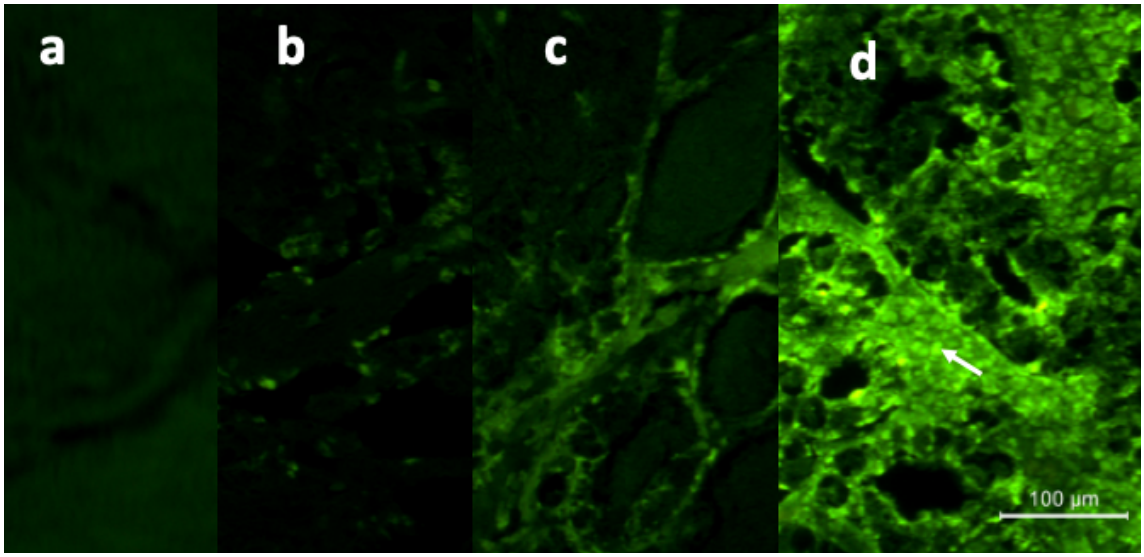


Fig 2.11 TUNEL assay on testis sections of a) unaffected b) stage 1, c) stage 2 and d) stage 3 JGC carp testes. TUNEL positive cells are marked by the green fluorescence indicating that they are apoptotic. Note, the increasing apoptotic signal intensity across stage 1-3, with none in unaffected tissue.

2.4.8 JGC in Females

Of the 6111 individuals examined 2106 were females, but none showed signs of JGC. However, one female exhibited a white multinodular tissue adjacent to the right ovary. Further observation revealed that this tissue did not have any resemblance to reproductive tissue. Dissimilar to JGC, this nodular vascularised mass was disconnected from the gonad. Moreover, histopathological observation did not show signs of apoptosis (Figure 2.12).

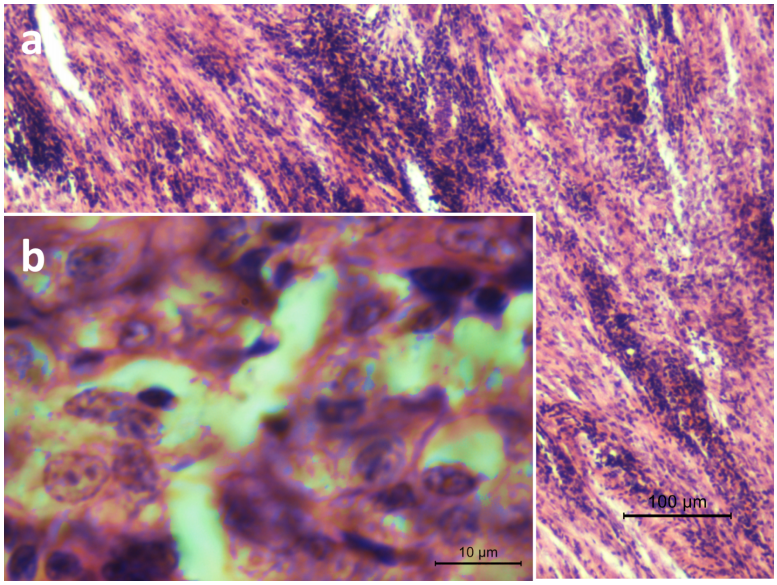


Fig 2.12 Histopathology of the solid neoplasia found in a female carp. b) Proliferating neoplastic cells exhibiting mitotic nucleus (Inset).

2.4.9 Incidence of gonadal abnormality in other fish in the lake

A total of 237 trout (97 males and 140 females), 67 golden galaxids (45 males and 22 females) and 30 eels that were caught (bycatch) during the routine carp management activity were also screened for gonadal abnormality. No bycatch displayed signs of gonad abnormality similar to the JGC condition encountered in the carp.

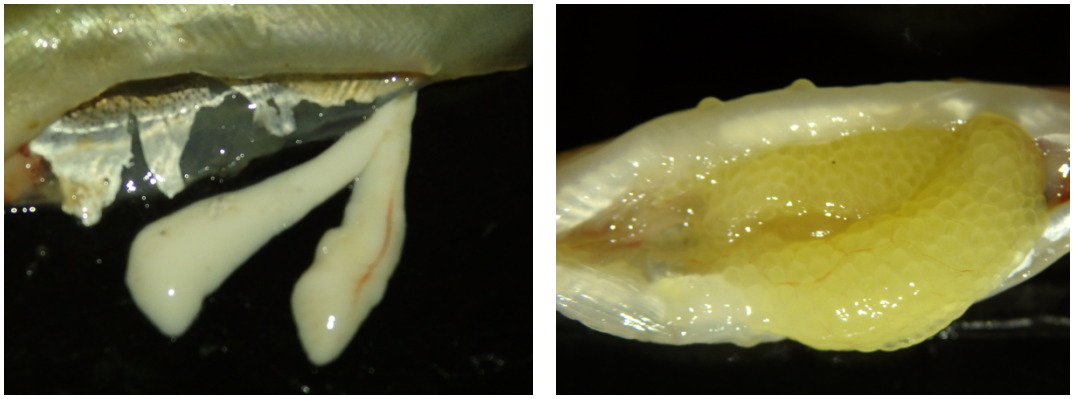


Fig 2.13 Representative healthy gonads of golden galaxids (Left-Testis and Right-ovary). No abnormalities resembling JGC were encountered.

2.5 Discussion

Discovering a naturally occurring abnormality in lower vertebrates frequently serves a unique opportunity to understand higher animal conditions including carcinogenesis (Couch, 1995; Grizzle and Goodwin, 2010). A large number of fish have been necropsied to understand the nature, pathogenicity and biological progression of testicular cancer and neoplasma, but only a few cases have been reported (Leatherland and Down, 2001). Largely, the number of affected animals encountered was low, thus precluding conclusive inferences and or limiting rigorous investigation. Moreover, these were predominantly associated with pollution. More recently, fish are emerging as unique models for human testicular carcinogenesis. For example, a mutation causing seminoma in zebrafish (Basten et al., 2013b) has been described as a model for a parallel human condition.

Historically, tumour and neoplasm related abnormalities in ornamental common carp (Nishikigoi) is well documented by Japanese breeders and is considered by aquatic animal practitioners as one of the models to understand gonadal abnormalities (Groff, 2004; Ishikawa and Takayama, 1977). Broadly, gonadal abnormality of common carp can be classified into three observable categories – (i) neoplasm-related abnormality in ornamental koi, known to occur infrequently (Ishikawa and Takayama, 1977; Ishikawa et al., 1976); (ii) *en masse* neoplasm and structural abnormality in inter-species (Down,

1984; Down and Leatherland, 1989; Granado-Lorencio et al., 1987; Sonstegard, 1977) and intra-species (Gupta and Meske, 1976) hybrids; and (iii) gonadal abnormality due to exposure of carcinogens/contaminants in laboratory experiments (Gimeno et al., 1998a; Gimeno et al., 1998b) or in the natural environment (Dickman and Steele, 1986). Interestingly, the gonad abnormality of carp encountered at Lake Sorell appears unique and different from any previous studies. It occurs in a very remote highland lake that is situated at the top of the river Clyde, with no known pollution history. Moreover, pollution could conceivably affect both male and female fish as well as other fish species in the lake. But, the condition is specific to males and thus far not encountered in trout, eels and golden galaxiids (the only other species of fish in the lake). The presence of bacteria, fungal hyphae, inclusion bodies or parasites were not found to be associated with the condition, suggesting its non-pathogenic aetiology. The contained status of Lake Sorell ensures absence of the goldfish (*Carassius auratus* L.) or other carp species with which it is known to hybridise nor do the affected animals show any morphological traits associated with hybrid animals.

This study also monitored the disease condition over several consecutive breeding seasons and examined almost every fish caught from the lake during the study period, making it the most comprehensive study of its kind. As the lake has a single cohort (2009 cohort) population, it was possible to monitor the progression of the disease including growth and maturity of the fish with greater certainty. The first appearance of the condition with onset of maturity in 2013-14 (8.6%) and its maintenance at comparable levels (7.9 %) in the following season (2014-15) suggests that the disease frequency was stable across the population but the increase was associated with maturity. This was further supported by relatively comparable proportion of male fish affected across seasons. For example, the percentage of males encountered with JGC in seasons 2013-14 and 2014-15 were 15.6 and 17.3 % respectively. Morphological analysis of the severity

stages also followed a similar stable trend of affected area albeit progressing to more advanced stages than before. For example, the proportion of stage 1 and stage 2 was the same (16%) in 2015 and 2016 respectively; indicating the fish with stage 1 condition in 2015 had progressed to stage 2 the following year. This was also supported by histological observations where severity categories retained elements that were typical of categories preceding or following the respective category i.e. demonstrating progression of severity with time/age.

The JGC condition appears unique morphologically with no comparable reports in fish or any other vertebrate species including humans. Natural manifestation, testis specificity, abnormal proliferation of Sertoli cells, followed by massive cell death, with no external symptoms other than expressing dilute/thin milt in early stages with rarely motile spermatozoa are hallmark of the condition. Generally, uncontrolled cell division in testicular tissue (both germ cell or non-germ cell cancer) produces a lump of homogeneous cells that results in a vascularised (sometimes nodular) neoplasm, that has been reported in many studies (Basten et al., 2013b; Dilworth et al., 1991; Romano et al., 2013; Young, 2005). Although Sertoli cell proliferation was observed in JGC tissue, massive cell death that occurs in later stages conceivably precludes a neoplasm formation is unique. It is possible that heightened proliferative signal during the development of JGC induces a state of cellular senescence in the later stages that finally triggers the cell death cycle in JGC tissue. In amniotic vertebrates the Sertoli cells do not retain the capability to proliferate whereas fish Sertoli cells can divide even in adult testes (Schulz et al., 2005). However, during the spermatogenesis they are produced in exact and required numbers (Schulz et al., 2010). This phenomenon of abnormal proliferation is common only in sex cord-stromal tumours/cancers, specifically in Sertoli cell only syndrome or Sertoli cell tumours (Dilworth et al., 1991; Sesterhenn et al., 2004). Indeed, sex cord/gonadal stromal tumours are a diverse group of neoplasm that includes Leydig

cell tumours, Sertoli cell tumours, granulosa cell tumours and tumours of the fibroma (Eble, 2004). In JGC affected tissue we failed to observe proliferation of cells other than the Sertoli cells, implying that condition is in part comparable to cord-stromal tumour. On the other hand, although spermatogenic arrest has been observed in certain types of non-cancer testicular degeneration, proliferation of any somatic cells was totally absent in those cases (Blanchard et al., 1996; Turner, 2007). As cancer is uncontrolled cell (in this instance Sertoli cells) growth with or without metastasis (WHO definition, Fact sheet, 2009) the observed JGC appears cancerous. This was also supported by upregulation of cancer marker in JGC tissue (Chapter 4). However, this cancerous nature of JGC needs further verification before using it as a model for Sertoli cell carcinogenesis.

The first histologically observable effect of JGC condition was the halt in production of spermatozoa followed by the mass proliferation of Sertoli cells. It is possible that malignancy of Sertoli cells triggered irreversible changes that immediately terminated support for the developing germ cells therefore spermatogenesis. Conceivably, this leads to loss of structural integrity of the testicular architecture and/or basic metabolic function of Sertoli cells activating the cell death mechanisms. However, as most of the cells are dead, the only remaining tissue is the fibrous materials that provide the solid support and hold the fluid in the blister like vacuoles. Nonetheless, continuous cell death makes the interstitial tissue thinner and weaker leading to fusion of the blisters to form large sacs in final stages.

Interestingly, histopathology of JGC also revealed chromatin condensation, karyopyknotic nuclei, and membrane blebbing of the dying cells (Figure 2.6b), concurring an apoptosis or autophagy driven cell death in later stages of JGC. This inference is further supported by a TUNEL assay that shows strong and wide spread positive signal across the affected tissue. The TUNEL assay is considered robust in

assessing DNA fragmentation due to cell death activation, particularly in testicular tissue (Pulkkanen et al., 2000). However, the extremely high and tissue-specific cell death without applying any chemo-toxic compounds, is unusual and not reported previously in any other species. No published reports are available to the best of our knowledge where uncontrolled cell division and mass apoptosis occurs in a same tissue that collectively results in complete degeneration of an organ, let alone its function. A follow up investigation focused on molecular regulation/mechanism of tumorigenesis and apoptosis in the affected animals could shed light on the etiology.

Successful therapy for testicular cancer generally depends on using various chemotherapeutic treatment which generally relies on drug induced apoptosis (Brown and Wouters, 1999; Oosterhuis et al., 1984; Spierings et al., 2003; Walker et al., 1987). The principles governing mechanisms of these drugs generally depends on their ability to cause DNA damage to the cancer cells as they are the malignant counterparts of their normal cells (Chaganti and Houldsworth, 2000; Skakkebaek et al., 1987). Overall, the FAS-FASL pathway are thought to be the crucial regulator in drug induced apoptosis in several tumour types (Debatin and Krammer, 2004). Particularly in testicular tissue FAS-FASL mechanism between Sertoli and germ cells are proposed to be the key regulator for apoptosis therefore Sertoli cells are considered as an excellent target for the chemotherapeutic toxicity (Boekelheide, 2005). Nevertheless, the absolute mechanism for the cancer cell death is not clear to date (Giuliano et al., 2006). As, primary investigation in JGC revealed the malignancy of the Sertoli cells in stage-1 JGC followed by massive cell apoptosis in the later stages, this could assist to discover novel mechanism of Sertoli cell malignancy and death of cancerous cells.

It appears that the condition is restricted to a carp population in Tasmania, as this has not been previously reported elsewhere. It is possible that the trait may have manifested as a

result of inbreeding as the founder population of the Tasmanian carp is thought to be very small—a handful of escaped bait fish used by trout anglers (Personal communication with IFS, Tasmania). As a result, it is entirely possible for this inbred strain to have some genetic incompatibility or instability predisposing the animals to JGC. In fact, inbred ornamental carp with restricted genetic diversity, produced by artificial mating also develop gonadal deformity (Ishikawa and Takayama, 1977; Sirri et al., 2010). Nonetheless, the sex specificity of the condition is intriguing and provides an unprecedented opportunity to investigate the sex-linked genetic mechanisms in this species and vertebrates at large.

Results of previous studies showed that fish with internal neoplasma display lethargy, abdominal swelling, and impaired motility (Granado-Lorencio et al., 1987; Hawkins et al., 1996; Raidal et al., 2006; Sirri et al., 2010). Hybrids with gonadal neoplasma showed large multinodular masses with abdominal swelling, sometimes with no effect on visceral organs (Down and Leatherland, 1989; Romano et al., 2013). Another case of encapsulated tumours with focal adhesion and no infiltrative growth to other organ were reported with impaired motility of animals leading to their death (Ishikawa and Takayama, 1977). Testicular tumours invading liver lobes and intestinal loops, leading to death of animals has also been reported in this species (Sirri et al., 2010). Similarly, a high mortality rate was observed in tumour bearing carp hybrids in Arrocampo Reservoir, Spain (Granado-Lorencio et al., 1987). In stark contrast to all the previous observations, the JGC condition is characterised by liquid filled blisters with no indication of metastasis. The affected fish do not show external symptoms of lethargy and no fish have been reported dead as a result of JGC. Additionally, the fish do not show any skin, kidney or gills lesions that resemble viral or pathogenic infection nor histopathology associated with bacterial/viral infection. However, production of dilute ‘milt’ with no motile spermatozoa in the severe condition render the animals sterile, perhaps mimicking an as

yet unknown male infertility condition related to cancer in other vertebrates, including humans.

So far the molecular mechanisms triggering Sertoli cell malignancy are largely unknown. The only model organism available to date are few transgenic lines of mouse model, however, each of the lines have their own limitations. For example, transgenic mice carrying a fusion gene composed of human Mullerian inhibiting substances SV40T linked to SV40 T antigen develops testicular tumour resembling Sertoli cell origin (Peschon et al., 1992), however, the tumour could not be studied in the model simply because the line could not be maintained as a result of reduced fertility. Similarly, mice lacking α- inhibin although develop mix gonadal carcinoma that effects both sexes and die at the age of 12 week due to severe cachexia like syndrome (Matzuk et al., 1992), precluding down-stream investigations. In another study, conditional deletion of Smad5 in mice results in metastatic mix cell tumour, however, both sexes were affected and their value in studying Sertoli cell tumour were limited (Pangas et al., 2008). Additionally, mouse model for Sertoli cell tumour established through stabilizing β-catenin in Sertoli cell although develops Sertoli cell tumours (Chang et al., 2009), proliferation of cells could not be linked as a causal effect of β-catenin stabilization. In contrast, to the mouse model, JGC is very easy to maintain, Sertoli cell specific, naturally occurring and prevalent in a high number, therefore has the potential to serve a model to study Sertoli cell malignancy.

2.6. Conclusion

In conclusion, based on habitat history, prevalence, gross morphology and histopathology, the JGC appears somewhat idiopathic. However, its exclusive association with the testes suggest male-linked genetic aetiology, that manifests with onset of puberty/maturity. While there are certainly differences between classical mammalian oncology and lower animal neoplasm, many lower vertebrates and indeed invertebrates

have served as a model for understanding higher animal carcinogenesis (Basten et al., 2013b; Couch, 1995). Common carp (*Cyprinus carpio*) is one of the best-known freshwater aquaculture teleosts with a long history of domestication and research, this unique condition in common carp could serve as an excellent model for understanding non-germ cell testicular degeneration, testicular development, interplay of onco-genes and apoptosis.

Chapter 3: Comparative growth, sperm quality and hormone levels of fish with Jelly-like gonad condition

Panel showing growth differences between normal (left column) and JGC (right column) affected males of same age-class/cohort.

3.1 Abstract

Sterile fish are gaining popularity for aquaculture and management activities as they minimise the impact on environment by biocontainment of selectively bred and genetically modified organisms (GMOs). Additionally, they maximise production by reducing early maturation and unwanted spawning during commercial aquaculture activity. While, many efforts have been made to sterilize fish using chemicals, genetic approach and surgical castration; they have proven not applicable for large scale operations and in many cases the fish suffer from growth deprivation to reduced physiological performance in the wild and culture conditions. In contrast, JGC, a recently identified and naturally occurring strain of carp exhibits a novel testicular condition (JGC) where the carp are unable to maintain reproductive capability i.e. sterile. As a first step towards evaluating its utility for aquaculture and management of carp, this chapter evaluated the effect of JGC on growth, reproductive and endocrine performance. Objectives were addressed by comparing association of biotic and abiotic factors (regression analysis and one-way ANOVA), comparing fish condition factor, mark recapture data analysis, assessing milt samples and hormone assay. While, JGC had a relationship to length, fishing year and few specific gears, no relationships were observed with months, strains and capture location. The results suggest that the JGC fish had 15.9, 12.3 and 6 % more body weight than their immature, male and female cohorts respectively. Data from mark-recaptured sub group of the cohorts indicated that the increased growth was associated with onset of the condition. Analysis of milt samples revealed a significant loss of spermatocrits in advanced stages of JGC with a presence of unusually high number of abnormal sperms. Additionally, CASA analysis of the normal and JGC milt revealed a significant loss of sperm motility associated with advanced JGC condition. Analysis of circulating hormone levels indicates a reduction of 11-ketotestosteron while increased production of Luteinising hormone was observed in

advanced stages. Collectively, the results indicated that JGC fish are close to sterile but exhibit better growth performance, therefore could be useful in large scale aquaculture and management applications. In addition to growth advantages, this could be an important system to investigate endocrine function, behaviour and reproductive biology.

3.2 Introduction

Aquaculture is one of the fastest growing sectors of food production, generating nearly 50 percent of total food fish (FAO, 2018). Worldwide production from aquaculture has doubled (both in value and volume) over the past decade (Naylor et al., 2001). Especially, the recent decline of ocean fisheries stocks has stimulated the growth of fish and shellfish farming. The aquaculture production not only includes native species but also exotics which constitute to 10-17% of the world's total aquaculture production (Garibaldi and Bartley, 1998; Shelton and Rothbard, 2006). While planned introductions of non-native species for farming bring economic benefits, most often a large number of introduced species contribute to ecological disruption, loss of native biodiversity and economic loss (Bernstein and Olson, 2001; Gozlan et al., 2010). Therefore, increased aquaculture activity of non-native species can lead to enhanced biological risks to native biodiversity.

Reproductively sterile farmed animals are recently gaining popularity as they offer excellent opportunity for minimizing the risk of environmental degradation during large scale aquaculture (Benfey, 2001) and management operations (Patil et al., 2015). Additionally, many farmed fish reach sexual maturity before attaining the market size, resulting in production and economic loss (Mair and Little, 1991; Roth et al., 2007). Typically, maturation results in diversion of energy from somatic to gonadal growth which often results in deterioration of flesh quality and animals become susceptible to disease and stress (Zohar, 1989). This, however, can be overcome by farming sterile fish, which not only ensures environmental security but also results in enhanced muscle

production and better fish health (Ali and Rao, 1989; Weber et al., 2014). Similarly, preventing unwanted spawning is also necessary in many commercially farmed species such as the common carp and tilapia that are prone to precautious maturation, particularly so in the tropics (Billard, 1999; Zambrano et al., 2006). Frequent spawning of cultured fish in production systems, result in undesirable stocking density and produce numerous stunted fish that are not market ready.

Sterile fish are also useful in management of feral pest fish populations. For example, carp eradication programs use 'Judas' fish technique to reveal the location of feral carp (Diggle et al., 2012; Taylor et al., 2012). Although this technique is proven to be successful for many invasive terrestrial (Cruz et al., 2009; Keegan et al., 1994; McIlroy and Gifford, 1997; Roy et al., 2002) and aquatic species (Diggle et al., 2012; Taylor et al., 2012), the threat of recruitment by the radio tagged 'Judas' animals is always present, especially when the population is low. For instance, contribution of Judas fish in targeted capture of carp in an eradication program at Lake Crescent, Tasmania was significant (63% of total catch), however, they were attributed to the catastrophic event of recruitment in Lake Sorell, Tasmania in 2009 (Diggle et al., 2012). Such unwanted recruitment risk can be minimised or avoided by using sterile animals to produce radio-tagged Judas fish (Patil et al., 2015).

The common methods of fish sterilization include interspecific hybridization (Bartley et al., 2000); use of chemical (Ali and Rao, 1989; Sangtian, 2013; Twohey et al., 2003), ploidy/chromosome manipulation (Arai, 2001; Benfey, 2001; Shelton, 2006), surgical sterilization (Patil et al., 2015), antisense technology (Hu et al., 2007; Uzbekova et al., 2000) and germ cell elimination through genetic engineering (Hsu et al., 2010; Hu et al., 2010; Wong and Collodi, 2013). However, most of the methods are not suitable for large-scale operation and each of the methods has their own limitation. For example, some

hybrids and polyploid strains of fish although mostly sterile, suffer from a growth deprivation and may not perform as well as the parental strains (Wong and Zohar, 2015). While the chemical castrations are transient with reproductive capability bouncing back with recovery of tissue (Patil et al., 2015; Sangtian, 2013), the genetic modifications attract regulatory and consumer concerns. Therefore, developing new strains and techniques with supressed reproductive capability, whilst optimising production and management applications with little or no regulatory and consumer concerns are highly desirable.

As outlined in chapter 2 a naturally occurring male-specific condition with compromised reproductive performance, but with no apparent health or growth consequences was identified and isolated form wild population of carp in the Tasmanian lakes. It appears that this condition dubbed as Jelly-like gonad condition (JGC) has not been previously encountered or reported from elsewhere in the world.

Common carp (*Cyprinus carpio*) is a chief farmed fish globally. Despite herbivore and bottom dwelling, the contribution of carp towards global fish supply and food security is enormous (Williams, 1997). For example, farmed common carp contributes nearly 14% of world aquaculture production (FAO, 2002). Carp exhibits a range of characteristics which provides them competitive advantages over most aquatic species (Koehn, 2004) and can quickly establish themselves as the dominant species after the introduction. Not surprisingly, these very hardy traits make them most desirable in production systems. However, due to its detrimental effect on native biodiversity, carp is considered as a serious vertebrate pest in many parts of the world including Australia (Braysher and Barrett, 2000; Group, 2000; Koehn, 2004).

As, both management and aquaculture of common carp is highly dependent on control of spawning/recruitment (reviewed in Chapter 1), this naturally occurring condition in Lake

Sorell could potentially open new door for a molecular approach for carp sterilization. As the abnormality is detected only in carp, it may be useful to develop a species-specific pest eradication tool. With an overarching objective of exploring the utility of the JGC carp for both its management and aquaculture, this study evaluated select biotic and abiotic factors associated with its growth, reproductive biology and habitat in Lake Sorell, Tasmania.

3.3 Materials and methods

3.3.1 Study area

Lake Sorell (42°06' S, 147°10' E) is a shallow highland lake of 50 km^2 with an average depth of 1.5 to 2.5m (at full supply level) situated in the south-east of Tasmania. The habitat consisted of barren areas of sediments, rocky shores and reefs (Taylor et al., 2012).

3.3.2 Source of capture data and sample collection

This study utilizes data collected by the carp eradication program over a period of 48 months, spanning 4 spawning seasons (2013-2017). Carp were captured by 4, 4.75 and 5" mesh gill nets. Location of capture, date of capture, total length, weight, and sex of each fish was recorded. Where available stage of gonad development and its weight was also recorded. Due to concerted carp management program at lake Sorrell no carp spawning occurred after the isolated event in 2009, contributed by a handful of adult carp that had remained following 10 years (1998-2009) of eradication efforts. Therefore, the population studied represents a single year class/cohort. Older remnants (50<) from previous spawning events could be easily identified and were excluded from the analysis.

3.3.3 Biological relatedness of the condition

To determine the spatial, temporal and biological relatedness of the condition, a total of 4594 fish that were caught between 2013/14-2016/17 fishing season were subjected to statistical analysis (fishing season is defined in chapter 2). As JGC is a binary variable

(Present/absent), a 'Binomial Logistic Regression' was carried out using R (version 3.3.3). Months (Jan-Dec), scale type (mirror or normal), sexual status of fish (male, female and immature), fishing season (5 years), fishing methods, length and weight were used as independent variable. The weight and length of the fish was represented arithmetically while the other variables were used as a factor. As a high correlation exists between the length and weight, one predictor variable was included at a time to overcome the multicollinearity in regression model. Overall, 40 different types of fishing gears were analysed in the study and 4 inch gill net (N4) was used as a base variable for regression. To understand the location preference of the JGC fish compared to normal male, a separate regression using normal male (N=885) and JGC male (N=187) caught in 2014 were used. Regression analyses was carried out with JGC as a dependent and locations as independent variables respectively. The lake was divided as eastern and western basin and the location data were represented as a dummy variable.

Growth (length and weight) of JGC and normal fish were compared by one-way analysis of variance (ANOVA $p<0.05$) followed by Tukey's-HSD (95% confidence level). Data were reported as means and standard errors. Fish with incomplete data in the database were excluded from analysis.

3.3.4 Fish condition factor

Condition factor was determined using Fulton's formula (Ricker, 1987):

$CF = Weight/Length^3$.

In all 3757 fish belonging to immature (n=233), female (n=1728), male (n=1359) and JGC (n=401) carp caught between 2014-17 were analysed. To observe the statistical difference between the groups (one-way ANOVA was performed followed by Tukey's HSD).

3.3.5 Growth analysis of the mark recapture fish

To observe the relative growth rate of JGC fish, data from fish that were released as part of a mark-recapture population estimate of the 2009 cohort, were analysed. In particular, growth data from 803 fish that were tagged and released during 1st -3rd of January 2012 (IFS, annual report 2011-12) was used. Fish fork length varied between 207 mm to 305mm at the time of tagging. Each fish received two pit tags with a unique number for re-identification. Mark-recapture data was obtained from IFS carp capture database. No tag fish captured before 140 days post-tagging event (Season 11/12) were found to be sexually mature therefore, were taken out from the analysis.

Unverified and incomplete data were not included in the analysis. Duration between tagging and recapture were calculated for each fish. Fish that were recaptured before 60 day post tagging were also deleted, as the sex for most of the fish were indeterminant during that period and the growth differences were not significant. Only male fish (includes JGC) were analysed.

The relative growth and the relative growth rate were calculated using the following formulae (Schreck and Moyle, 1990) –

Relative growth (%) = $(L_f-L_i)\times100 / L_i$ and Relative growth rate (%) = $(L_f-L_i)\times100/(t \times L_i)$

Where, L_f = Final fork length; L_i = Initial fork length; t = Time difference between tagging

The relative growth was plotted against time (Day difference between catch and release) and linier regression trend line fitted separately for male and JGC fish. The relative growth rate for male and JGC fish was calculated as mean and standard error for each fishing season.

3.3.6 Milt Sampling

Semen collection were performed following the standard protocol described for common carp (Bastami et al., 2010). Before collecting the milt sample, the urogenital area of the male carp was wiped dry with a paper towel to ensure contamination (from urine, water or faeces) free milt. Milt was expelled by a gentle pressure on the abdomen, collected using a pipet, immediately transferred to a micro centrifuge tube and a subsample was transferred in naturally buffered formalin (NBF) for later analysis. Milt analyses were limited to normal males and two categories of JGC fish—primary and advanced.

3.3.7 Spermatocrit assay

Spermatocrit density (calculated as a ratio of packed sperm cells to the volume of semen × 100) was used to measure the concentration of sperm cells (Agarwal and Raghuvanshi, 2009; Butts et al., 2010). About 70% of the micro haematocrit capillary tubes (10 μL, Length 41 mm) were filled with undiluted common carp milt, in duplicate. The capillary tubes were sealed with clay sealant at both ends and centrifuged for 2 minutes at 6000×g using a haematocrit centrifuge, at room temperature. Percentage of packed cells in the capillary tube was measured using a haematocrit reader and the results documented. The mean value for both measurements were calculated for each male and analysed statistically (One way ANOVA).

3.3.8 Analysis of sperm morphometry

Morphological observation of the sperm were conducted following standard procedures (Butts et al., 2010; Silva et al., 2015). Semen from a total of 33 samples (n=11 in each category) from three different groups of fish (normal male, preliminary JGC and advanced JGC) were assessed. Briefly, duplicated smears were prepared using formalin fixed (natural buffered) semen samples (diluted 1:500 for controls and 1:20 for JGC). Slides were air dried, washed in absolute ethanol (5min), stained with 3% Bengal rose

and air dried. A total of 300 spermatozoa from each slide (600 in total per male) were assessed using a combination of light and phase contrast microscopy. However, in very advanced JGC samples (n=2) only 50 sperm/slide were evaluated due to insufficient sperm content. Structural abnormality of the sperm were primarily classified into three groups- a) head defects (detached head, micro head, macro head), b) middle piece defect and c) tail defects (very coiled tail, detached tail) (Silva et al., 2015).

3.3.9 Assessment of spermatozoa velocity

Velocity of actively swimming spermatozoa was determined by computer assisted sperm analysis (CASA) using ImageJ, following standard protocol (Adair, 2015). As carp milt contains a high density of sperm (e.g. up to 30 x 109 spermatozoa/ml), serial dilution of freshly collected milt were carried out for all the control fish milt using 0.1M PBS (phosphate buffered saline). Milt samples from JGC fish were not diluted as the sperm density was low and suitable for direct analysis. A sperm activation solution was prepared by filtering (0.2 micron) tank water that was used for activating all the sperm samples analysed. For spermatozoa activation, 5 µl of milt sample (known dilution from control or undiluted samples from JGC) were mixed well with 50 µl of activation solution. Immediately, 3 µl of the activated samples was carefully transferred to the semen analysis chamber (Leja®, Nieuw-Vennep, The Netherlands). Time between sperm activation and start of video recording was recorded. Any recording delays that exceeded 20 second post activation were discarded, and the assay repeated. All the analyses were carried out at ambient temperature. Video were captured using an eyepiece camera (Dino-Lite AM4023) fitted with AMCap recording software under phase contrast microscope (400x magnification). Samples of normal male and primary and advanced JGC male (n=10 for each category) were recorded in duplicates until all the samples had less than 1% motility. All the videos were converted to suitable file format (*.avi, 10 frames/second) before

uploading to ImageJ. Pairwise differences of the group means were compared using one-way ANOVA followed by Tukey's-HSD (95% FDR) in R.

3.3.10 Hormone assays

Blood from recently captured and euthanised carp was drawn by caudal puncture using a 90 IU-heparinised 3 ml-syringe fitted with 21 gauge needle (Sangtian, 2013). Roughly, 1ml blood was collected from each fish and temporarily stored on ice until processed. Chilled blood was centrifuged (3000g, 5 min, 4^{o}c) and plasma stored in liquid nitrogen and transferred to a -80^{o}C freezer for prolonged storage. Unique specimen ID was assigned for each sample collected during the sampling period.

Circulating levels of 11-keto-testosteron (11-KT) and luteinizing hormone (LH) in carp plasma were assessed using commercially available fish specific enzyme linked immunosorbent assay (ELISA) kits 11-KT-582751 (Cayman, USA) and LH-MBS-031319 (MyBioSource, USA) as per manufactures instructions and those described before for this species (Adair et al., 2018; Patil et al., 2015).

Briefly, all reagents and solutions were thawed at room temperature before the assay. Samples were diluted 1:200 with EIA buffer for the 11-KT assay while used undiluted for the LH assay as the level of LH remained within the detectable range of the kit.

For the 11-KT assay, diluted samples were aliquoted into coated wells as per the manufacturer's instructions, incubated as required and allowed to develop for 100 minutes on an orbital shaker away from light. All the samples were run in duplicates with a minimum of two blank, two non-specific binding and three maximum binding controls as well as an eight-point duplicated calibration standard. Following incubation, the wells were washed, rinsed, reaction developed and concentration measured using a colorimetric

plate reader (Synergy HT, Biotech) at 412 nm. The assay results were calculated with the built-in software using the calculated standard curve.

Similarly, for the LH assay, all the samples, standards and blanks were assayed. Maximum binding and total activity analyses were omitted from assay as these were not required. Incubation was done at 37^{o}C for 60 mins. After washing the plate five times chromogen A and B were added, incubated away from light source in 37^{o}C for 15 min. Data were generated using a colorimetric plate reader (Synergy HT, Biotech).

3.4 Results

3.4.1 Relationship of JGC to select biological and environmental factors

Regression analysis of 4594 fish caught between 2013/14-2016/17 indicated a significant relationship between JGC condition and selected predictors. Among the independent variables, length ($p < 2e\text{-}16$) and weight ($p < 2e\text{-}16$) showed a significantly strong positive correlation with the JGC condition. Predictably, JGC had a significant relationship with male carp ($z = 12.38$, $p < 2e\text{-}16$). In contrast, month of capture did not show significant difference at the 95% confidence level. Similarly, the mirror population ($z = 0.49$, $p = 0.63$) did not show any significant difference in JGC index compared to normal males. None of the gears with the exception of n5 (5" gill mono net), mm5 (5" multi-mono gill net) and 5h (5" gill net) showed any significant propensity to selectively capture JGC fish when compared to 4" gill net. A separate regression analysis using 811 male and JGC fish (all captured 2014) showed that there was no difference ($\beta = -0.789$. $p = 0.245$) in association of the condition with the eastern and western basins of Lake Sorell. Table 3.1 contains a brief summary of the regression analyses, including standard errors and probability for all the predictors. More details of the regression result and schematic relationship between the predictors are represented in Appendix A.

Coefficients	Estimate	Std. Error	z value	Pr (>|z|)
Intercept	-1.06E+01	1.38E+00	-7.709	1.27E-14***
Sex (male)	4.84E+00	3.91E-01	12.377	< 2e-16***
Weight	2.48E-03	2.89E-04	8.597	< 2e-16***
Length	1.86E-02	2.08E-03	8.931	< 2e-16***
Season 13/14	1.62E+00	6.18E-01	2.626	0.00864**
Season 14/15	2.08E+00	6.35E-01	3.276	0.00105**
Season15/16	3.12E+00	6.48E-01	4.818	1.45E-06***
Season16/17	3.82E+00	6.52E-01	5.852	4.85E-09***
Month	-	-	-	>0.05
Mirror	1.15E-01	2.36E-01	0.486	0.62729
Method n5	7.32E-01	2.40E-01	3.052	0.00228**
Method n5h	5.82E+00	1.39E+00	4.173	3.00E-05***
Other methods	-	-	-	>0.05

Table 3.1: Summary of the regression analysis between JGC and key independent variables. Individual months and most of the fishing gears were insignificant therefore are presented collectively as months and other methods respectively. Length and weight were analysed in separate analysis to avoid multicollinearity problem. Significant codes: '***' 0.001 '**' 0.01 '*' 0.05.

As the regression analysis revealed a significant relationship between JGC to growth measures (length and weight), one-way ANOVA with Tukey's HSD was conducted for

a quantification of the growth differences between groups. Overall, pairwise comparison confirmed a significantly increased growth (both length and weight) of JGC fish compared to normal males in each of the 4 different seasons (Figure 3.1). When compared with female and immature fish, JGC fish had higher fork length initially (season 13/14-15/16) however, found to be similar in the later season (16/17). Collectively, JGC fish had 15.9, 12.3 and 6 % more body weight than immature, male and female fish respectively (Figure 3.2a). Similarly, JGC fish were 7.5, 4.7 and 3.7 percent longer than the immature, male and female fish respectively (Figure 3.2b).

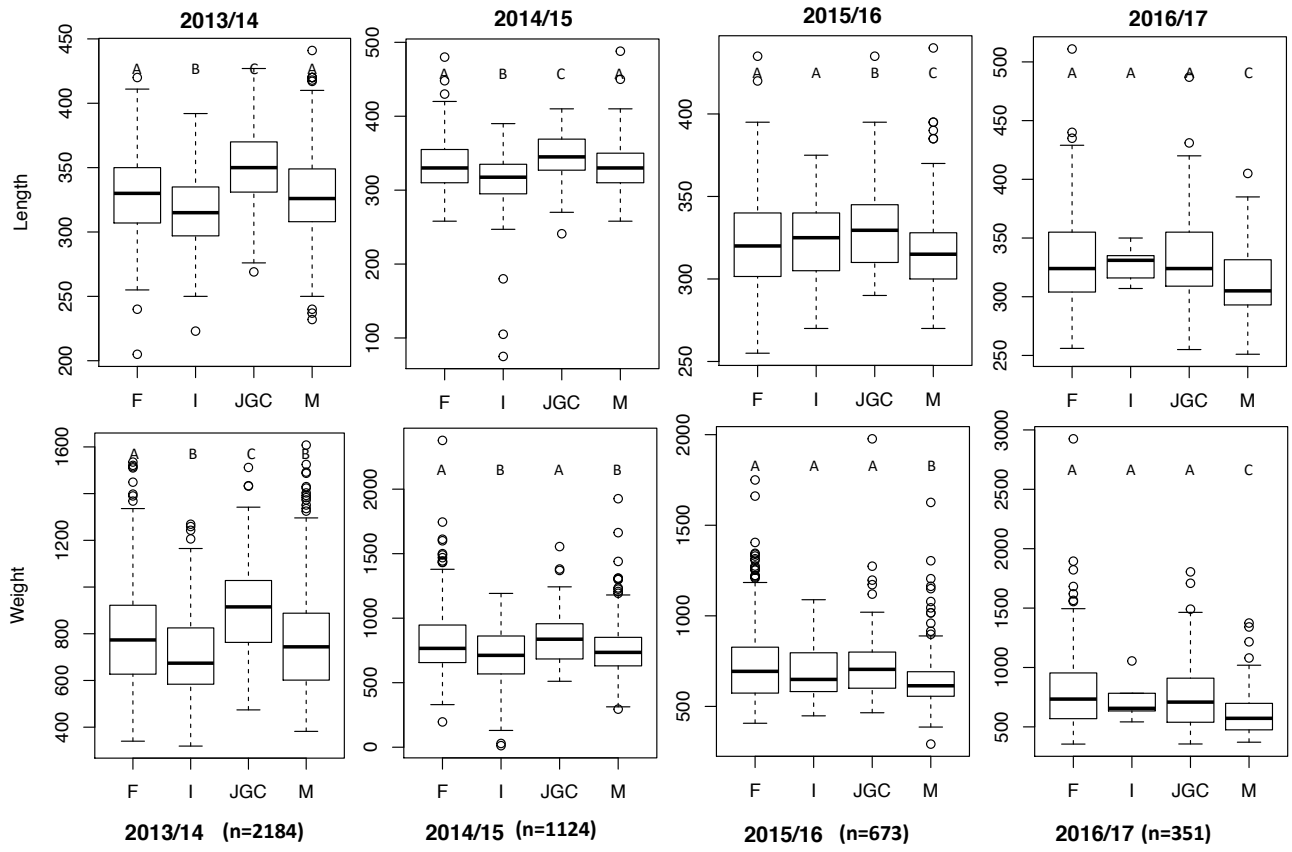


Fig 3.1: One way ANOVA and Tukey's HSD showing significantly (at 95% confidence level) enhanced growth of JGC fish compared to normal males in four different fishing years. Note: F=Female, I=Immature, M=Normal male and JGC=Jelly like condition.

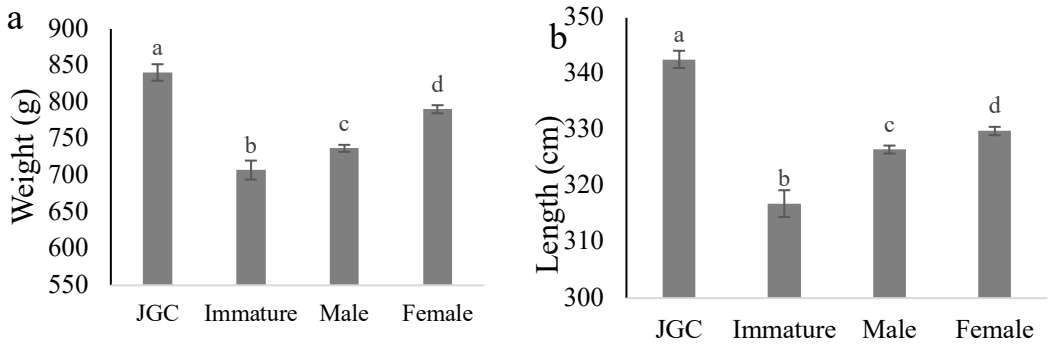


Fig 3.2: Growth of JGC carp compared to immature, normal male and normal female cohorts captured between 2013/14 and 2016/17 fishing season indicating JGC fish has increased growth. Data presented as mean (± standard error) length and body weight.

3.4.2 Fish condition factor

Observation of fish condition indicated no difference between the male and JGC fish (One way ANOVA, Tukey's HSD). However, the condition of male and JGC fish were significantly lower than those of females and immature fish (Figure 3.3).

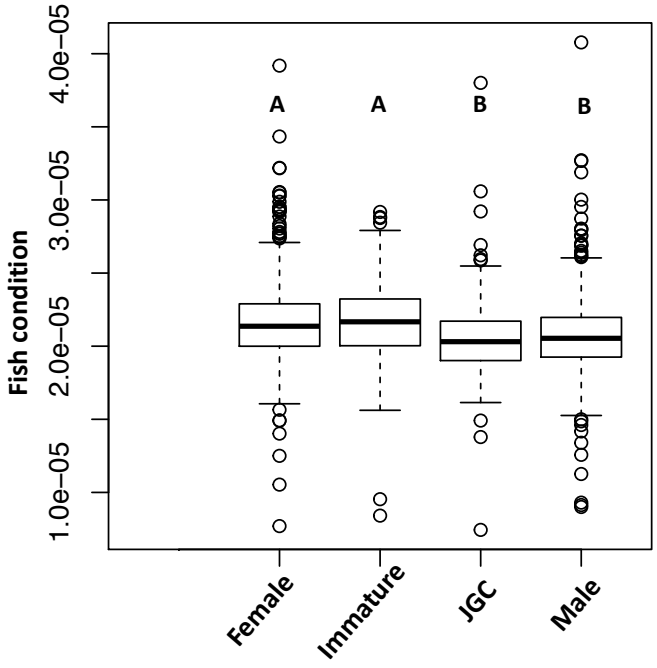
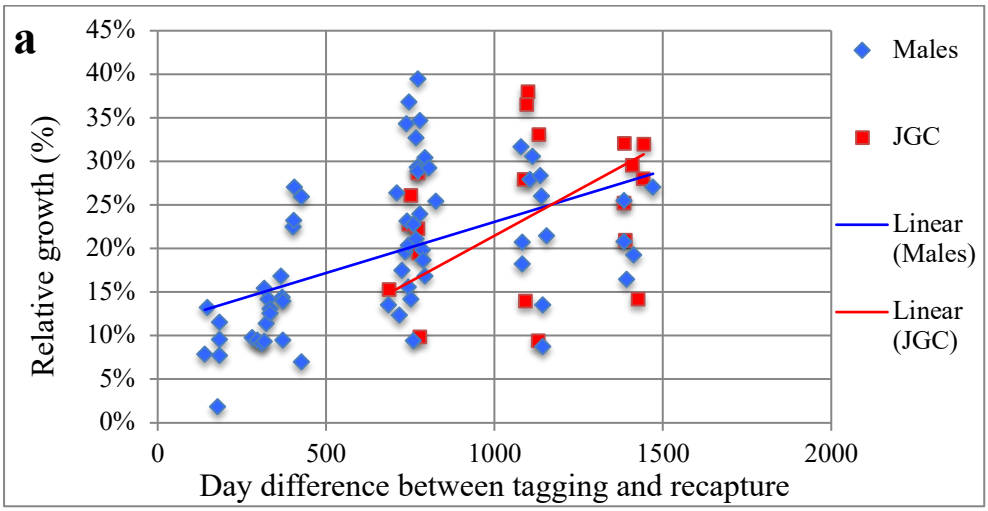


Fig 3.3: Fish condition factor

3.4.3 Growth analysis of the mark-recaptured fish

Data analysis over the five consecutive fishing seasons (11/12-15/16) showed an overall increase of relative growth of all the tagged fishes. The first JGC fish was recaptured 687 days after the tagging. Relative growth of the JGC fishes was lower than the normal males at the onset (around 600 days post tagging) of the condition, (Figure 3.4a). Over the next two fishing seasons (Day 1000-1200 and day 1300-1500) a sustained growth was observed in the JGC fish, which resulted a sharper regression line. In contrast, the growth of normal males was slower than the JGC fish in the later years.

Similar to the relative growth rate, mean absolute growth rate of the JGC fish was lower than the normal control male fish before the onset of JGC condition, however, in the later seasons JGC fish exhibited higher growth rate than the normal fish (Figure 3.4b).



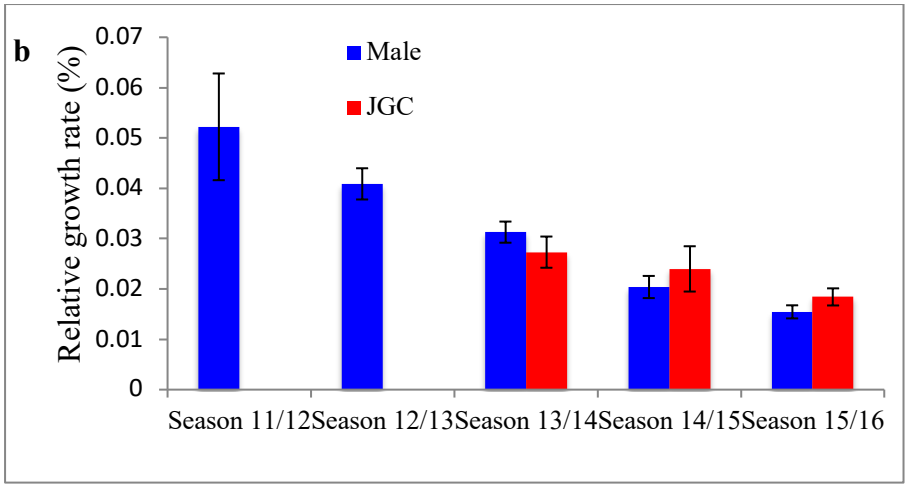


Fig 3.4: Relative growth (a) and mean relative growth rate (b) of mark-recaptured fish from 0 (date Jan 2012)-1600 (date May 2016) days.

3.4.4 Semen evaluation

Semen samples from all normal males were viscous and creamy white irrespective of their volume obtained. In contrast, the semen samples from the primary JGC fish were translucent and less viscous in consistency progressing to clear and water-like in the advanced JGC fish Figure 3.5.

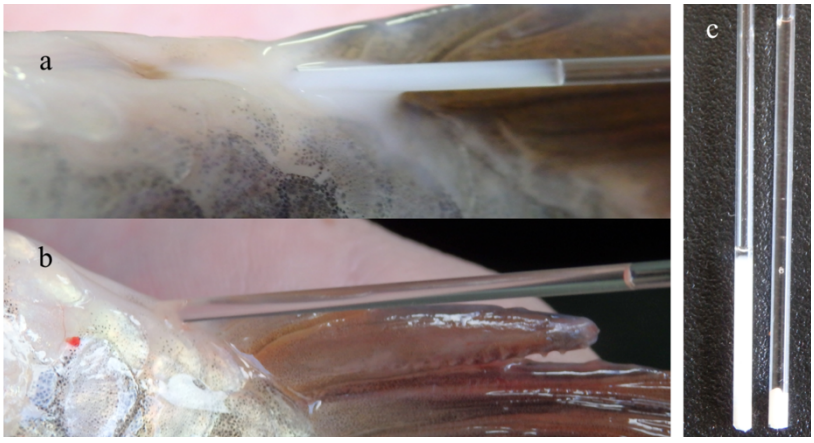


Fig 3.5 Semen from (a) healthy and (b) JGC fish. (c) comparison of spermatocrit content of healthy (left) and JGC (right) fish.

3.4.5 Spermatocrit assay

High density packed cells (on average ~75%) were observed in normal milt while the percentage packed cells was low in JGC milt (Figure 3.5 c). Fish that was in their

preliminary stage of the condition had on average ~54% of packed cells while in the advanced condition the average percentage of packed cells was below 10%. Observation of some very advanced JGC indicated that the spermatocrit density could be as low as 1.3%. Mean difference between the groups were significant ($p < 1.6e-11$) at 95% confidence level (ANOVA, Tukey's HSD) (Figure 3.6a)

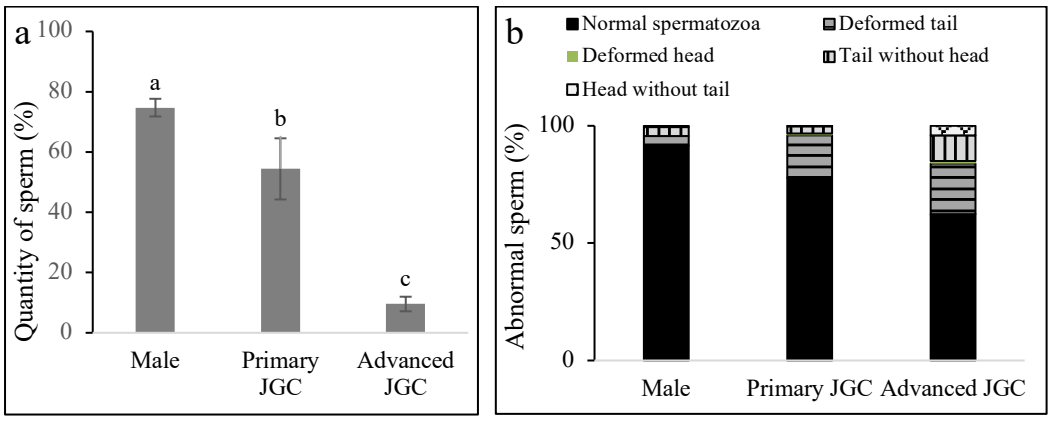


Fig 3.6 Quantity (a) and quality (b) of spermatozoa in normal and JGC carp. Both quantity and quality of spermatozoa in JGC affected fish was significantly compromised.

3.4.6 Sperm abnormality

Morphological analysis of the sperm indicated that higher sperm abnormality was associated with the JGC condition. Normal male semen contained on average 91.6% healthy sperm whereas it was only 77.9%, 62.4% in primary and advanced JGC males (Fig 3.6b). Sperm deformity were mostly associated with the head and tail with no defect detected in the middle section. Tail abnormality (4% in control, 18-22% in JGC) mostly consisted of varying degree of coiled tails (99.3%) with a few folded tails (Figure 3.7 b). No other types of tail abnormality (e.g. multiple tail) were detected. The proportion of deformed head (0.07% in control, ~1% in JGC) were lower than the deformed tail, and mostly consisted of abnormally small head or micro head with no presence of macro head. The percentages of sperm with a missing head (~3%) or tail (<0.6%) were similar in control and primary JGC but, increased sharply in advanced JGC (missing head 11%,

missing tail 4%) (Figure 3.6b). Examples of sperm abnormality detected in JGC males is presented at Figure. 3.7.

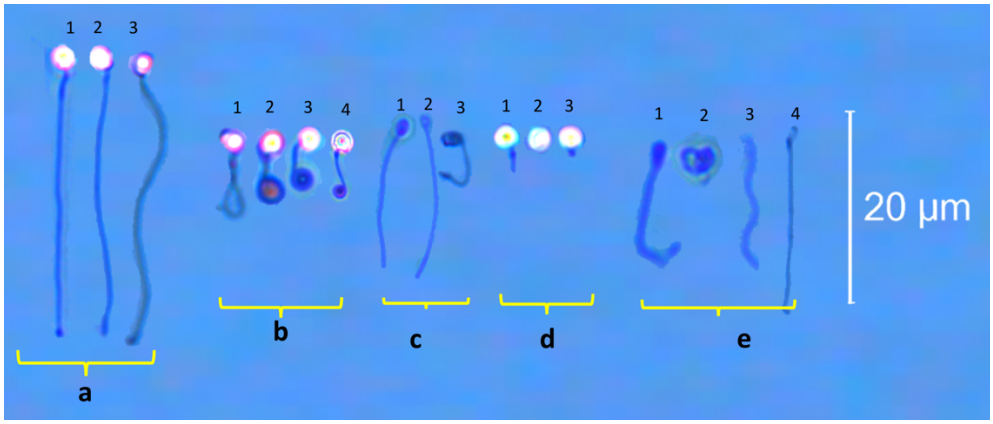


Fig 3.7 Photograph of(a) healthy and (b, c, d and e) abnormal sperm observed in JGC and normal carp. Note: b = Tail abnormality (folded tail[1] and coiled tail[2-4]), c= head abnormality (micro head[1,2] and abnormally shaped head[3]), d= missing tail, e= missing head. The photographs were acquired using phase contrast microscopy.

3.4.7 Sperm motility

CASA analysis of normal and JGC milt showed a significant reduction of sperm quality parameter associated with JGC males. Specifically, the percentage of motile spermatozoa started to reduce with onset of JGC condition (68% on average) than the normal male (91%) which further dropped to 17% on average at the advanced conditions. CASA analysis on some advanced JGC fish showed that content of motile spermatozoa could be as low as 0%. Similarly, all the sperm velocity parameters (VCL, VAP and VSL) of the advanced JGC fish were significantly lower than the control. Additionally, the total distances (progression) travelled by the activated spermatozoa, wobble and total semen activity time were also significantly low for advanced JGC fish. No significant difference was observed with LIN and BCF between the groups. Result for the CASA analysis is provided at Figure 3.8.

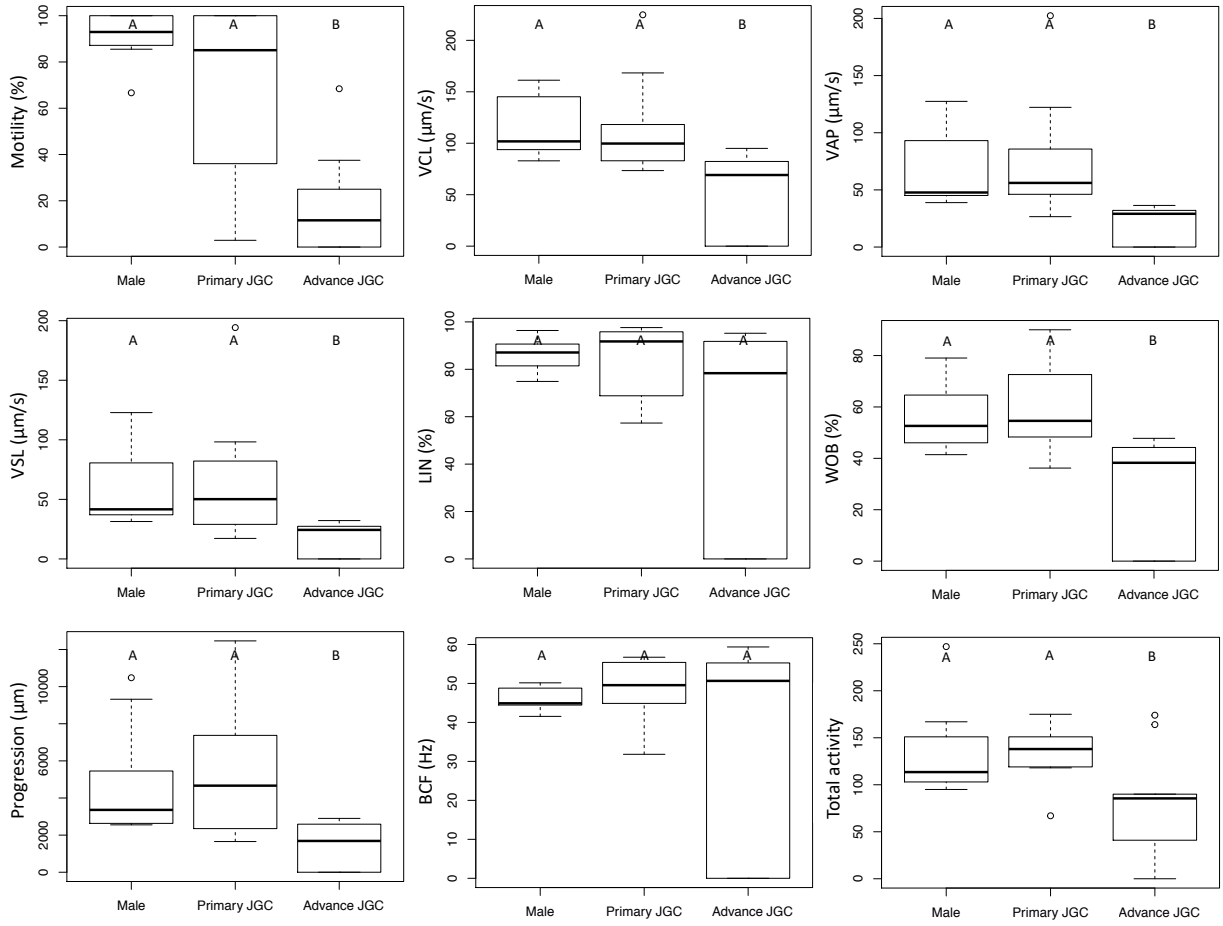


Fig 3.8 Mean (± standard error) value for the sperm quality parameter of normal, primary JGC and advanced JGC males. Note: VCL, curvilinear velocity; VAP, average path velocity; VSL, straight line velocity; LIN, linearity; WOB, wobble; BCF, beat cross frequency.

3.4.8 Circulating hormone level

Mean hormone level of carp serum were compared between the females, normal males and four stages of JGC. The level of 11-KT in females and all stages of JGC were lower than the males ($p<0.05$). In contrast, the mean LH level for the normal male and female were almost similar. In JGC group, a drop in plasma LH level was observed in stage 1 however, it gradually increased in later stages of JGC (Figure 3.9).

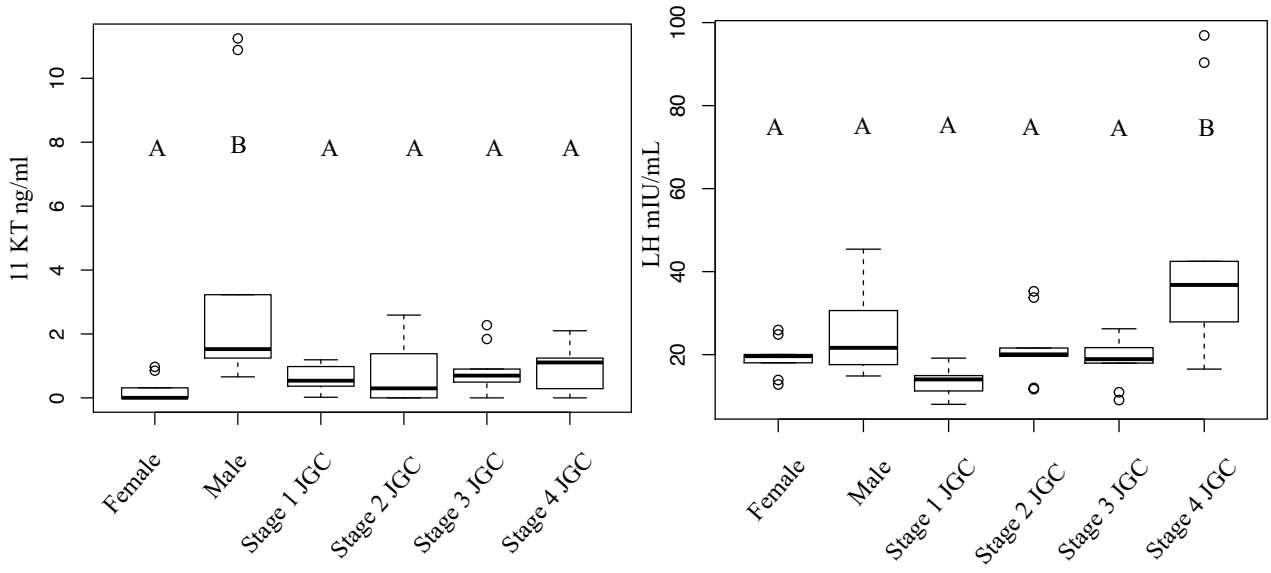


Fig 3.9 Concentration of circulatory 11-KT and LH in female, male and stages 1-4 of JGC carp.

3.5 Discussion

Reproduction in higher animals is dependent on successful completion of gametogenesis. However, its suppression such as in sterile fish can increase somatic growth (Wolters et al., 1982), resulting in better flesh and low mortality rate (Gjerde, 1986; Zohar, 1989). This occurs primarily due to shift of energy from reproduction to somatic growth (Wolters et al., 1982). While, reports are available to supress reproduction in order to acquire enhanced growth (Basavaraju et al., 2002; Oppedal et al., 2003; Wolters et al., 1982) and reduced environmental impact (Basavaraju et al., 2002; Wong and Zohar, 2015), effect of naturally occurring gonadal abnormality on growth and physiology of the wild fish has never been studied. For example, gonadal abnormality due to environment deterioration (Dickman and Steele, 1986; Harries et al., 1997; Jobling et al., 1998) or genetic predisposition (Bittner, 2009; Down et al., 1988; Ishikawa et al., 1976) have been studied on several occasions, however, their consequences on growth and reproductive performance have not been described.

As a novel gonadal abnormality of common carp the prevalence,

morphological/histological characteristics and cellular changes of JGC condition have been described in chapter 2. Discussed here is the relationship of the JGC condition to select biotic and abiotic factors and its relevance to carp management and aquaculture.

3.5.1 Relationship of the condition to sex, months, scale type, fishing season and location of capture

In concurrence with 'male only' occurrence of the condition (Chapter 2), a significant relationship was found with the sex of the fish (Table 3.1). No significant relationship of months to JGC variable was detected, indicating no seasonal effect on the condition. Similarly, no effect of scale type (scaled and mirror carp) indicated the condition was distributed evenly across the two strains (scaled and mirror variants) of carp in Lake Sorell. Interestingly, fishing year showed a relationship to the JGC variable. Consistent with this observation, prevalence analysis of JGC fish indicated that the severity of JGC fish is increasing each year (chapter 2), which may explain the relationship of the condition to fishing year. Moreover, no relation to lake location indicated the condition was encountered with equal frequency across the eastern and western basin of the lake and hence the JGC fish have comparable behaviour to normal males such as searching food, aggregation and habitat preference among others. Similarly, this kind of result has been described for the sterile common carp (Patil et al., 2015).

3.5.2 JGC carp exhibit accelerated growth and are prone to gear selectivity

The present study confirmed that the growth (both length and weight) of JGC fish was significantly greater than the normal male, female and immature fish of the same cohort. These results were consistent across four consecutive breeding seasons/years. The growth effect was more obvious with length than the weight measurement, particularly so later in the fishing season. One possible explanation could be the female carp gained weight sharply owing to gonadal growth or accumulating gonad mass in the later years (i.e

increased/ the GSI (Lavado et al., 2004)) that was reflected in the fish condition analysis (i.e. female had better condition than male and JGC).

Growth of the fish is an ongoing process. However, it is generally known that growth significantly slows down in mature fish (i.e. with age) compared to the juveniles when the growth occurs at a much faster rate (Goeger, 1989). Although, as to when this enhanced growth of JGC fish is triggered is not clear; the mark recaptured dataset suggested that this occurs only after the initiation of the JGC condition i.e. (Figure 3.4b).

The increased growth rate observed in JGC carp makes them attractive for culture operations. This could be a result of bypassing energy from reproduction which has been documented in many cases of sterile fish (Bartley et al., 2000; Wolters et al., 1982). A follow up study detailing the interplay of physiology, behaviour (aggressiveness and sluggishness) and molecular changes in gonadal tissue could shed more light on the underlying mechanisms.

Interestingly, few fishing gears namely mm5, n5 and n5h exhibited selective fishing for JGC. Interestingly, all of them were $5^{//}$ mesh gill net which is the largest mesh gill net used in that lake (personal communication). One possible explanation could be, as the JGC fish were on average larger than the normal male fish, they were more susceptible to capture by larger mesh gill net while normal males escaped capture by $5^{//}$ nets. Such size targeted gear selectivity has been described from commercial fisheries data for many species (Hansen et al., 1997; McClanahan and Mangi, 2004).

Growth of common carp is highly variable especially in temperate regions. For example, after the first year, fish size can vary from 105 to 109mm while can be up to 300mm in the second year (Crivelli, 1981; Prochelle and Campos, 1985). Additionally, with supplemental feeding with cereals, daily growth can be as high as 2-4% of the body

weight (Flajšhans and Hulata, 2007). However, this growth can be considerably low in colder regions where they take up to 5 years to reach 350-400 mm fork length (Fernández-Delgado, 1990). The data from Tasmanian carp indicate the growth rate is even slower. This could be due to a combination of reasons ranging from continuous disturbance and exclusion of feeding habitat by the IFS eradication program confounded by the low temperatures at Lake Sorell. Significantly, loss of habitat and food supply has a negative effect on fish growth (Jones, 1986).

3.5.3 JGC fish are nearly sterile

At the very outset, reproductive success depends on fertilization of the eggs by healthy spermatozoa. As species with external fertilization, successful spawning of carp is directly dependent on the quality of gamete. It is well documented that sperm concentration, ejaculation volume, sperm morphology, sperm motility, longevity of sperm and sometimes chemical composition (Taborsky, 1998); individually or collectively determine the potential for fertility of male gametes. Observation in the study indicates the quality parameter for the carp semen dropped sharply in JGC carp. For example, semen obtained from normal male were creamy white indicating a high concentration of sperm cells (spermatocrit ~75 ± 3 %), while the content of cells dropped quickly (spermatocrit content ~54± 10%) at the start of the condition leading to a translucent watery semen and becoming almost clear (spermatocrit content 9.5 ± 2.4%) at the advanced stage. Similarly, the content of the healthy spermatozoa dropped (15-32%) significantly with JGC condition. Deformities such as sperm with a broken tail would lead to reduced motility (Rurangwa et al., 1998) of sperm rendering the animals reproductively sterile (Van Look, 2001). Additionally, a large proportion of JGC sperm had coiled tail, conceivably further reducing their ability to fertilise eggs. Coiled tail sperm is considered as one of the most common sperm defects resulting in reduced fertility (Chenoweth, 2005). A higher proportion of sperm deformity observed in JGC

carp may provide an opportunity to explore the cause and effect of such deficiencies in sperm morphology.

Spermatocrit content and the sperm velocity of the carp milt may slightly vary between the males and across the season (Lubzens et al., 1997; Rurangwa et al., 2004). Quality parameters for the normal male sperm observed in this study were found comparable to those previously reported in *Cyprinus carpio* (Christ et al., 1996). However, a mark reduction of quality parameters for JGC fish indicate that the JGC milt may not be suitable for successful fertilisation, at least in a natural habitat. Observed reduced sperm velocity in JGC milt (e.g. VCL, VAP, VSL and WOB) could be linked to structural abnormalities such as the coiled sperm tail.

In most freshwater fish with external fertilization, spermatozoa remain quiescent in the genital track and seminal fluid (Poupard et al., 1998) due to the high osmolality (Morisawa et al., 1983). It is generally known that the difference in K^+ ion concentration triggers an osmotic pressure that initiates the movement of fish spermatozoa upon ejaculation (Billard et al., 1995). However, once activated, the sperm remains motile for only a short period (Kime et al., 2001). The significantly reduced sperm motility of JGC carp from 68% in early JGC condition to very low (18%) in advanced stages, compared with normal male (91%) suggest that the fish are slowly but surely becoming functionally sterile. It is possible that the sperm in JGC fish are prematurely activated due to the changed physiological milieu of the testis (i.e. fluid accumulation). This is unlikely as any premature activation, is likely to render them immobile. For example, complete cessation of sperm movement has been reported due to the contact with external fluid (e.g. urine) as it results in a reduced ATP content in fish milt (Poupard et al., 1998).

3.5.4 Endocrine response of JGC fish

There are two male sex steroids produced in the testis; Testosterone (in higher vertebrate) and 11-ketotestestorone (in fish) (Borg, 1994). Spermatogenesis in vertebrates generally is supported by the active production of these androgens by the Leydig cells in testis (Komen et al., 1995). It is generally known that LH regulates production of these steroids through a feedback regulations, although the exact mode has remained obscure (Schulz et al., 2001). The LH level for the JGC fish dropped quickly after the disease induction, gradually rising in advanced stages, with a concurrent decline in 11-KT levels, suggesting a feedback response by the testis. Such feedback induction of LH following dropping 11-KT levels has been previously reported in fish (Okuzawa, 2002).

In mammals, LH stimulates the androgen production, whereas, FSH directs the regulation of Sertoli cell functions (Steinberger, 1971). Unlike mammals, the teleost gonadotropin regulation is complex as both gonadotropins (LH and FSH) have prominent steroidogenic potency (Planas and Swanson, 1995). Specifically, investigation in salmonids indicates FSH and LH pathways are interlinked (Oba et al., 1999). While this investigation provides evidences of disruption in the steroid production process (i.e reduced KT-levels) in JGC testes and its feedback regulation of LH, FSH was not investigated in this study.

While it is commonly known that androgen levels and gonadotrophin production are interlinked, many of the aspects of the regulatory mechanisms are still unknown in teleosts partly because of the process is more complex than mammals and also because there is a lack of suitable fish system/model (for review see Schulz and Miura, 2002). Considering the Sertoli cell proliferation, testicular degeneration and associated hormonal imbalance in the JGC carp, it could lend itself as an ideal system for investigating multiple aspects of steroidogenic pathways and their regulation. Laboratory experiments to understand endocrine function often require surgical removal of

reproductive organs (Cunninghum et al., 1978; Robertson, 1958) which often causes mortality and stress in experimental animals. However, JGC fish provide advantages as manual removal of the testes is not required. Moreover, rescue experiments through the treatment with GnRH analogues, gonadotropins, androgens, anti-estrogen, aromatase inhibitors could be feasible (Liu and Handelsman, 2003).

3.5.5 Potential use of JGC fish in aquaculture and fisheries management

Usually gonadal abnormality is associated with lethargy, physical deformity and mortality in fish (Chapter 1). However, in JGC carp related mortality, lethargy or invasive neoplastic growth were not observed. Indeed, the animals showed an improved growth performance than the control fishes over a sustained period of time without impacting the fish condition. Additionally, culture of JGC can also prevent unwanted spawning which is undesirable in polyculture of common carp (Basavaraju et al., 2002).

As a result of its invasive nature, eradication programs are underway in many parts of the world including in Australia (Diggle et al., 2012). However, complete removal of carp from large waterbodies is extremely difficult once they have established. While the management operation of this species often requires the use of 'Judas' carp (Diggle et al., 2012; Taylor et al., 2012) for targeting feeding and spawning aggregations, the risk of spawning by the Judas fish is always present. While effort has been made to use surgically sterilised common carp (Patil et al., 2015) as Judas fish, they were proven challenging to generate. As, naturally occurring near sterile condition, JGC carp offer themselves as alternative candidates for generating sterile Judas fish without the risk of contributing to recruitment

3.6 Conclusion

The present study provides the first documentation of enhanced growth that is triggered

by a testicular abnormality in wild caught fish. Unwanted spawning and early maturation are a common obstacle during commercial aquaculture operation as they not only have an adverse effect on growth but could lead to a higher stocking density. As JGC fish are near sterile and exhibit better growth rate, they might be valuable as an aquaculture trait, especially during large scale production. Their potential value to aquaculture will be further discussed in chapter 5. In addition of growth benefit, JGC fish could also be valuable for a research system for understanding reproductive performance, endocrine response in teleosts and Judas fish production for management of pest. However, as a near sterile fish production of the line could be difficult, hence future investigation is necessary.

Chapter 4: Comparative transcriptome analysis of a rare testicular abnormality in the common carp *Cyprinus carpio* (L).

4.1 Abstract

Reproductive pathology accounts for 50% of the total sterility in men. Infertility can be caused by many reasons including germ cell abnormality, testicular tissue degeneration, cancer, loss of gametogenesis and endocrine system failure, that predominantly have a genetic basis. Animal models are increasingly gaining popularity to investigate many of the human disorders as many of the molecular functions are conserved across the vertebrates. With a view to understand what causes Jelly-like gonad condition (JGC) carp (chapter 2), this study investigated the comparative transcriptome profile of JGC and normal carp. Whole transcriptome deep sequencing with ribozero treatment to ensure the complete detection of any involvement of long non-coding RNA and the protein coding genes was undertaken. Gene ontology analysis was carried out to identify genetic pathways that are affected by the condition. Based on RNAseq analysis, a total of 7,129 genes, encompassing 130 pathways, were found differentially expressed at a significant level ($p<0.05$) between the normal and JGC testis. Chromosome separation, cell morphogenesis, nucleic acid activity, spermatogenesis, gonad development, steroid production and apoptosis pathways were significantly altered in JGC testis, indicating a complete loss of testicular morphology and function that render the animals near sterile. Further rationalisation using NCBI and KEGG database identified 40 genes with a central role in developing the condition. Interestingly, BLAST characterization of the differentially expressed genes indicated that 26 of these genes are novel (no BLAST homology). Further investigations are necessary to identify the potential roles these novel genes may play in sterility, non-germ cell testicular cancer and spermatogenesis. The role of the pathways in trigger, progress and persistence of the condition has also been discussed. Overall, results of this study could be useful for understanding molecular basis of the testicular abnormality, related sterility and cancer in general.

4.2 Introduction

As spermatogenesis and testicular function is a conserved and co-ordinated process, any abnormality in the testis could result in a complete or partial sterility of animals, including in humans with life-risk in many cases (Petersen et al., 1998). For example, about 16-17% of the human couples are unable to conceive naturally after over 18 months of intimacy due to various degrees of infertility (Thonneau et al., 1991) with at least half of the cases attributed to male infertility (Benkhalifa et al., 2014; Mosher, 1988). Conspicuously, there is significant relationship between sperm abnormality and malignancy leading to infertility (Honig et al., 1994; Raman et al., 2005). It is generally known that subfertility and testicular cancer shares key etiological genesis (Lass et al., 1998; Petersen et al., 1999). Despite a wide range of studies on testicular abnormality, infertility, cancer and degenerative condition in humans and model organisms (Basten et al., 2013b; Ellis et al., 2004; Raman et al., 2005; Turner, 2007) our understanding on how these are related and manifest is poor.

It is commonly known that instability in genome and somatic mutations are one of the major hallmarks of cancer (Hanahan and Weinberg, 2011), sperm abnormality (Yatsenko et al., 2010) and infertility (Benkhalifa et al., 2014). Such changes always trigger an irregular expression of wide ranges of protein coding genes, microRNA (miRNA) and noncoding RNA (ncRNA) (Iorio and Croce, 2009). Increasingly, conventional approaches (e.g. karyotyping and Fluorescent In-situ Hybridization) are proving insufficient to investigate the role of these genes in infertility investigation (Benkhalifa et al., 2014) as they are limited in scope and capacity to asses multiple candidates in parallel. However, recent advances in a wide range of molecular methods have accelerated the understanding of male infertility in humans and other organisms. For example, investigations using sequence specific mutation/microdeletion (Dieterich et al., 2007; Foresta et al., 2000; Tiepolo and Zuffardi, 1976), autosomal gene targeting (Basten

et al., 2013b; Jamsai and O'bryan, 2011; Litchfield et al., 2016), transcriptomic (Guerrero-Bosagna et al., 2013; Montjean et al., 2012) and proteomic (Martínez-Heredia et al., 2008) techniques are enabling robust investigations into cause and effects of infertility and cancer of reproductive organs. Such molecular analyses facilitate a ready insight into the cancer and related infertility mechanism as well as identify novel genes that may have a role in triggering the whole process. Further characterization of such genes either as a biomarker or as a candidate for cancer through cancer cell/tissue expression profiling has yielded numerous clues eventually leading to improved diagnosis, subtyping/classification, research and treatment (Alizadeh et al., 2000; Brunner et al., 2012; Network, 2012; Nielsen et al., 2002). Such experiments have also led to the discovery of unique antigens (for example, cancer testis antigens—CT antigens) which are considered promising target molecules for vaccines (Scanlan et al., 2002; Simpson et al., 2005).

Model organism are valuable to understand vital and common features of many cancer forms as well as to study their pharmacodynamics and pathogenicity (Basten et al., 2013b; Oosterhuis and Looijenga, 2005). Despite their limitations, animal models provide an opportunity to speed up the cumbersome process of elucidating pathogenesis and drug discovery (Sharpless and DePinho, 2006; Talmadge et al., 2007). Animal models such as mouse (Arap et al., 1998; Olive et al., 2009; Sharpless and DePinho, 2006), dog (Khanna et al., 2006), Zebrafish (Amatruda et al., 2002; Basten et al., 2013b; Feitsma and Cuppen, 2008), killifish (Couch, 1995), carp (Leatherland and Sonstegard, 1978; Leatherland and Down, 2001) have contributed to understanding many cancer forms and related disorders. Historically, study of naturally occurring rare conditions have repeatedly shed light on effects, cause and treatment of many widely known animal and human diseases (Jamshidi et al., 2015).

Recently, we have discovered a novel and naturally occurring testicular abnormality in a cohort of wild carp (Chapter 2) in a large inland lake (Lake Sorell, Tasmania), where the affected animals develop “Jelly like gonad condition (JGC)”. Morphology suggests, complex pathology with signs of uncontrolled non-germ cell proliferation followed by cell death, infertility due to arrest of spermatogenesis leading to poor gamete production and eventual degeneration of testicle. Interestingly, affected animal exhibit improved growth (Chapter 3). Analyses so far indicate that this condition is unique but shares features with cancer related infertility (Chapter 2). Unlike other rare incidents, JGC occurs naturally at high prevalence (Chapter 2) thus ensuring adequate supply of samples for rigours investigation. However what triggers and regulates the entire process is completely unknown to date. With a view to capture both early triggers and the progress of JGC this study undertook differential transcription analysis of early stage JGC and healthy carp testis.

4.3 Materials and methods

4.3.1 Sample collection

The fish used in the study were captured as part of an integrated carp management program by Inland Fisheries Services (IFS), Tasmania at Lake Sorell (42°06' S, 147°10' E). To capture both early and late transcriptomic changes, testicular tissue from two freshly dead biological replicates of healthy (MC11 and MC 16) and early stage (stage 1) JGC (JG 11 and JG12) were obtained during the peak summer period and preserved in RNAlater for 10 hours before transferring to -80^{0}C.

4.3.2 RNA extraction

Total RNA was extracted from control and diseased tissue using RNeasy Plus mini kit (Qiagen GmbH, Hilden, Germany) using standard protocol. Briefly frozen RNAlater stabilized tissue was homogenised thoroughly in 650 µl of Buffer RLT, centrifuged and

the supernatant was collected. Equal volume of 70% ethanol was added to the supernatant and mixed thoroughly. The mixed solution was then loaded on to a RNEasy spin column, centrifuged and the flowthrough discarded. After washing the column with wash buffer (RW1), 80 µl of DNAse1(2.73 Kunitz units/µl) were added to the spin column and incubated at 37°C for 30 minutes. Another round of washing with RW1 and RLT buffer were performed to wash excess DNAse1. RNAse free water was used to elute the RNA and the extracted RNA was immediately transferred to ice. A small aliquot of extracted RNA was used for RNA QC with remaining RNA stored at -80°C. DNA contamination was measured with a Qubit dsDNA HS kit (ThermoFisher scientific, USA).

4.3.3 RNA QC, library preparation and sequencing

Quality and integrity of the RNA was measured with a Fragment Analyser ™ (Advanced Analytical Technologies, Inc.). cDNA was prepared using the total RNA after Ribo-zero treatment (to include detection of ncRNAs). A total of 1µg of high-quality total RNA from each sample was used for cDNA synthesis. A sequencing library was prepared by random fragmentation of the cDNA, followed by 5′ and 3′ adapter ligation using TruSeq Stranded Human-Mouse-Rat Total RNA LT Sample Prep Kit (Illumina, USA). Quality control was performed at each step during library preparation. Adapter ligated sequences were then amplified, purified and sequenced (Paired-end, read length 101, 6GB/sample (in compressed file format) using a HiSeq 2500 platform (Macrogen, South Korea).

4.3.4 Reference genome, annotation and analysis platform

Reference genome (Accession number GCF_000951615.1) and annotation for common carp (*Cyprinus carpio*) was downloaded from NCBI FTP site (ftp://ftp.ncbi.nih.gov/). Expression of the genes was analysed using publicly available web-based platform Galaxy (https://usegalaxy.org/). Raw reads (FASTQ file) including the reference genome and annotation was uploaded using Cyberduck (v 5.1.0)

4.3.5 Quality control of the reads

Raw data was checked and the integrity of each raw files was checked with a 'md5sum' algorithm before uploading on analysis platform. Reads were checked thoroughly for each of the samples to ensure good quality sequencing. Specifically, Q20/Q30 (%) score was checked, size of the inserts was visualised and filtered with a quality cut-off 20 and with a minimum percentage of 90. Finally, another FastQC was performed to ensure data meets the criteria set for the analyses. Insert size matrix, mean insert size and standard deviation of the insert were calculated using Galaxy toolkit Picard_Collect Insert Size Metrics, (v 1.136.0) for each sample for downstream use.

4.3.6 Mapping reads to reference genome

First the high quality paired-end filtered reads were mapped against the common carp (*Cyprinus carpio*) reference genome using the alignment program TopHat (v 2.1.0) (Trapnell et al., 2012) implementing "-v" alignment mode for Bowtie (v 1.1.2) (Langmead et al., 2009) for initial read mapping. The maximum number of mismatches allowed in each segment alignment for read mapping were set at 2 base pairs. Additionally, calculated mean inner distances and standard deviation were used in each of the mapping stages. To obtain best mapping of the reads, specific junction data with gene annotation model was included. However, mapping was not limited to junction data with a view to detect novel contigs expressed in JGC/controls. Quality of the read mapping was assessed statistically and visualised with Integrated Genome Browser (desktop IGB-9.0.0).

4.3.7 Transcript assembly, estimation of abundance and differential expression

Assembly of the reads into transcripts, estimation of their relative abundance and differential expression was performed using cufflinks (v 2.2.1.0). At least 95% of the

bases were required to align to the reference genome with maximum of two mismatches. Assembled transcriptome were visualised using IGB (desktop) to ensure quality of the Cufflinks output. Individual RNAseq libraries were merged into a master transcriptome file using Cuffmerge (v2.2.1.0). Count data (as BAM files) was used as input into Cuffdiff (v2.2.1.3) with false discovery rate set at 0.05, multi-read correct option and classic-FPKM (Fragments Per Kilobase of transcript per Million mapped reads) normalization. Significant differentially-expressed genes were filtered for further analysis. Visual outputs were made using Cummerbund in RStudio from the SQLite database created by the Cuffdiff output.

4.3.8 Gene Ontology term analysis

Genes (mRNA) that were differentially expressed between the control and diseased testes were converted to Zebrafish orthologs except for the ncRNAs. Gene short name were assigned for each of the genes. To annotate highest number of genes and assign appropriate gene ortholog, a combination of NCBI (genes and nucleotide) and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases were used. Further refining was done using NCBI BLAST tools where necessary. Pathway analyses were conducted for the first 5130 genes that were differentially expressed ($p \leq 0.002$). GO analysis were performed on Cytoscape (v3.5.1) using the plugins ClueGO (v2.3.4) and CluePedia (v1.3.4). Pathways and ontologies of the differentially expressed genes were analysed against Danio rerio GO-Biological Process, GO-Cellular Component, GO- Immune system process, GO-Molecular Function, Interpro Protein domains, KEGG and REACTOME databases. Enrichment analysis was performed using 'two-sided hypergeometric test' with Bonferroni step down correction/normalization. The threshold for the FDR value was set at $p \leq 0.05$. Clustering of the ontologies were made using AutoAnnotate. Duplicated results were curated. Gene expression of each of the nodes

was carefully screened. Enriched terms were grouped into related broad categories based on the shared genes/features.

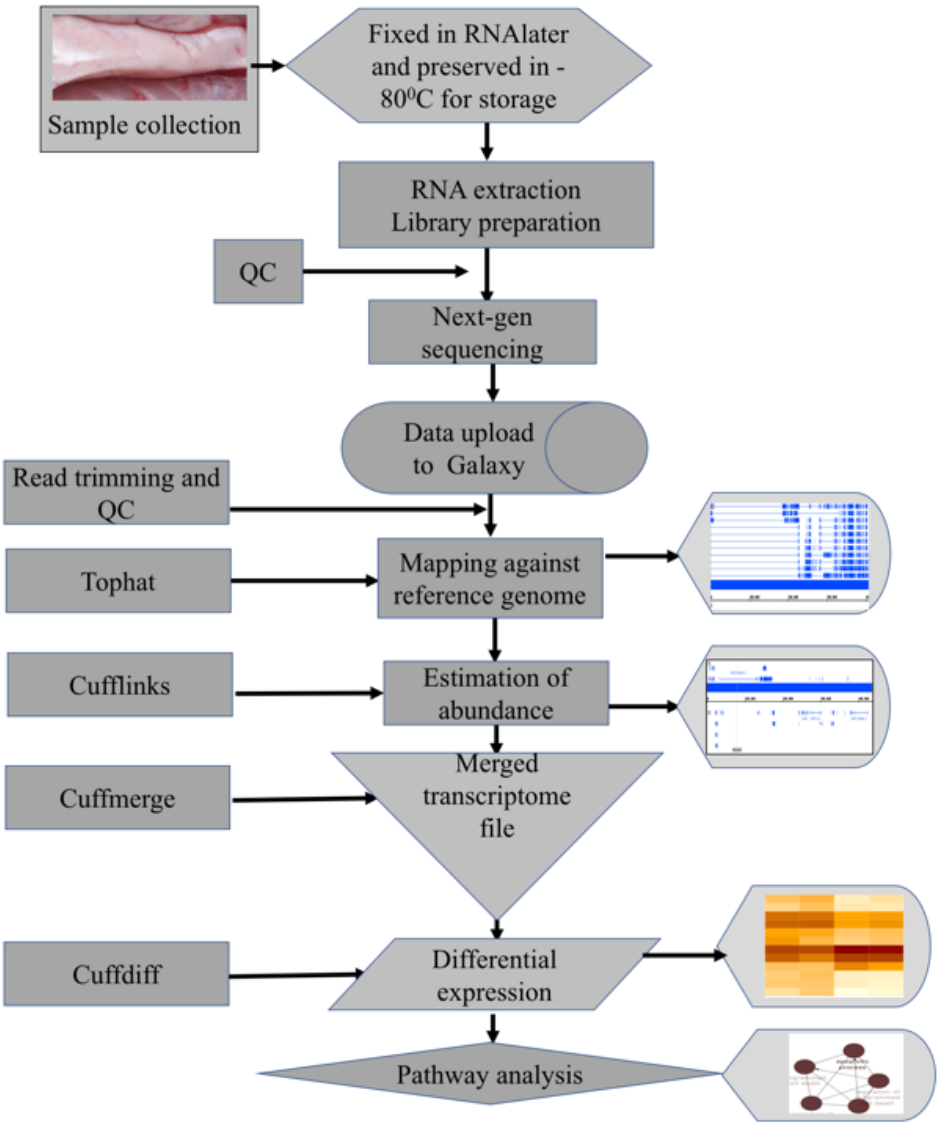


Fig 4.1 A schematic diagram summarising key steps employed in RNAseq analyses.

4.4 Results

4.4.1 RNA extraction, QC and sequencing

All the samples sent for NGS sequencing had RNA Integrity Number (RIN) score above 7 (Appendix B1). Presence of residual DNA was less than 1pg/µl for all samples. Sequencing coverage was on average 9 Gbp / sample. A summary of the number of the bases, number of reads, GC(%) content Q20(%) and Q30(%) scores are presented in

Appendix-Table B1. After uploading to analyses pipeline, 7-10% of the reads were filtered out due to poor quality. Another round of FastQC confirmed that the reads met essential statistical requirements (Appendix B3).

4.4.2 Mapping of the genome

The mean inner distances between the mate pairs were used as -10 throughout the experiment as the average mean insert size was found to be 190 bp (an overall view of insert size is provided in Appendix 4d). On average 75.9% pairs of cleaned reads were mapped against the reference genome of the common carp (a detail mapping summary is provided in Appendix TableB2).

4.4.3 Characterization of RNAseq data

Comparative global gene expression of common carp testicular tissue (control and JGC) revealed the expression of a total of 88,324 genes in 149,871 isoforms. Among the total number of expressed genes 7,129 were differentially expressed (DEGs) in JGC compared to the control. Based on the RNA expression pattern differentially expressed genes could be classified into two broad groups; 1) genes that are either upregulated or downregulated than in control, 2) genes that are totally absent either in control or JGC (uniquely expressed). Overall, 4,348 genes were upregulated whereas 2,781 genes were downregulated in JGC tissue than healthy testis. Among the DEGs, a total of 5,157 (~72%), 325 (~4.5%) and 420 (~6%) sequences were characterized using NCBI as protein coding, ncRNA and pseudo genes respectively. As the common carp genome is significantly large and relatively less annotated (compared to human or Zebrafish), a significantly large number of differentially expressed sequences (1,227; ~17%) are as yet unknown to current knowledge (i.e. remain unnamed). A summary of the differentially expressed genes is provided in Figure 4.2.

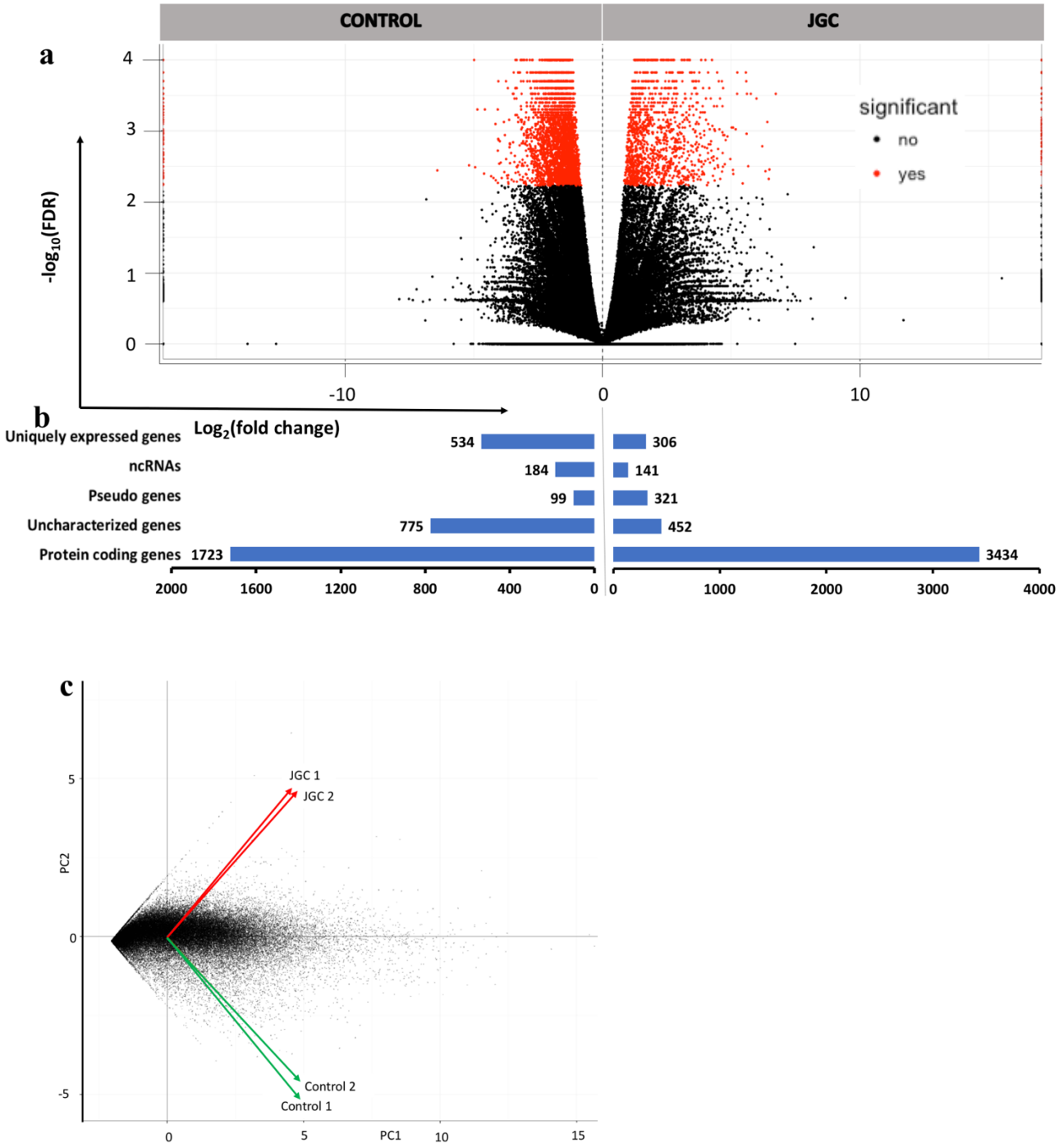


Fig 4.2: Summary of differentially expressed genes in JGC and control tissue. a) Volcano plot showing significance (FDR<0.05) log fold changes with affected (red) and unaffected (black) genes, b) categories with number of differentially expressed genes and (c) PCA plot representing the homogeneity among the replicates are presented.

Among the differentially expressed genes, a total of 40 candidate genes were identified as directly associated with JGC. In brief, this ranking was made according to the highest

fold changes, significance, FPKM value (>25) and unique pattern of expression (e.g. absent either in control or JGC). Loci containing housekeeping genes with high and consistent pattern of expression (e.g. calcium binding, histone, ubiquitin genes, collagen genes etc.) were taken out from this list. However, a select few of these housekeeping genes are presented separately (section 4.4.5.9). The highest fold change was observed in a ncRNA (XLOC_027968) i.e. downregulated in JGC. However, an uncharacterized locus (ID. XLOC_079856) was found highly expressed in control gonad but totally undetectable in JGC condition. While most (37) of the top ranked 40 genes (Table 4.1) were downregulated, three (*apoeb*, *scg2a* and XLOC_080469) were upregulated in JGC.

Interestingly, some of the contigs with high fold changes were unusually long (>5000bp). In contrast, among the 20 unknown sequences 9 were very small (<200bp) and they all expressed uniquely either in control or JGC.

Table 4.1: A list of the top 40 differentially expressed transcripts in JGC and control carp testis.

Gene short name/XLOC ID	Description	FPKM Control	FPKM JGC	Log fold changes	P value
XLOC_027968	lncRNA	557.6	3.1	-7.5	5E-5
spaca9a	sperm acrosome associated 9	213.6	1.7	-6.9	5E-5
tex36	testis-expressed protein 36	92.9	0.9	-6.7	3E-4
XLOC_061677**	ncRNA/gene	223.3	2.6	-6.4	7.5E-4
XLOC_066038	lncRNA	342.5	4.6	-6.2	1.7E-3
XLOC_061405	Novel	70.0	1.0	-6.2	5E-5

XLOC_015325	Novel	31.7	0.5	-6	3.5E-4
*odf3b***	outer dense fibre protein 3B	132.7	2.3	-5.8	5E-5
XLOC_034135	Novel	195.6	4.2	-5.5	5E-5
XLOC_033292	mRNA /Function not known	201.0	4.7	-5.4	5E-5
spaca9b	sperm acrosome associated 9	214.2	5.2	-5.4	5E-5
XLOC_034599	Novel	367.2	10.0	-5.2	5E-5
ropn1l	ropporin-1-like	62.7	1.8	-5.1	5E-5
pvalb1	parvalbumin-like	1240.1	35.8	-5.1	5E-5
rsph3	radial spoke head protein 3 homolog	119.6	4.0	-4.9	5E-5
*pvalb7***	parvalbumin-7	1114.7	42.8	-4.7	5E-5
XLOC_072908	lncRNA	288.3	12.1	-4.6	3.5E-5
dynlrb1	dynein light chain roadblock-type 1	196.0	14.6	-3.8	5E-5
XLOC_080832	Novel	51.3	3.9	-3.7	1.7E-3
spata4	spermatogenesis-associated protein 4-like	129.6	11.1	-3.5	5E-5
h4	Histone H4	3901.7	489.9	-3.0	5E-5
XLOC_056699	Novel	253.1	39.9	-2.7	2E-3
h2afy2	histone H2A-like	9785.5	1886.8	-2.4	5E-5
XLOC_080469	Novel	5.0	92.6	4.2	5E-5
apoeb	apolipoprotein Eb-like	78.7	3688.1	5.6	5E-5

scg2a	secretogranin-2-like	0.5	27.5	5.8	5E-5
XLOC_029803	Novel	0	30.1	OEJ	5E-5
XLOC_058575*	Novel	0	167.0	OEJ	5E-5
XLOC_079279	Novel	0	502.2	OEJ	5E-5
XLOC_011176	Novel	0	30.7	OEJ	2E-4
XLOC_055937*	Novel	0	46.6	OEJ	2E-4
XLOC_068976*	Novel	0	41.0	OEJ	3.5E-4
XLOC_084050*	Novel	0	29.7	OEJ	1.3E-3
XLOC_013676*	Novel	0	207.5	OEJ	2.2E-3
XLOC_025022*	Novel	0	137.5	OEJ	2.2E-3
XLOC_015677	lncRNA	0	98.9	OEJ	5E-3
XLOC_079856	Novel	8054.2	0	OEHT	5E-5
XLOC_074817*	Novel	100.7	0	OEHT	5E-5
XLOC_035212*	Novel	159.9	0	OEHT	5E-5
XLOC_061873*	Novel	164.4	0	OEHT	5E-5

* =very short sequence (<200bp).

Novel = No BLAST homology

** very long sequence (>3000 bp) may contain multiple genes in a cluster, expressing together).

OEJ = Genes only expressed in JGC, OEHT = Only expressed in healthy testis.

4.4.4 Pathway analysis

4.4.4.1 Gene ontology (GO) analysis

Gene ontology analysis of differentially expressed genes revealed a total of 130 statistically overrepresented terms in 3 functional groups- 1) Biological Processes, 2) Cellular Components and 3) Molecular Functions. However, GO analysis performed against the immune system processes did not yield any ontologies at statistically significant level (FDR<0.05). In brief, among the 130 enriched GO terms (Figure 4.3), most (n =115) were related to biological processes with a few related to cellular components (n = 7) and molecular functions (n = 8).

As per Bonferroni step down normalization, chromosome separation (GO:0051304) was the most affected pathway (p=8.80E-53, category no 5, Figure 4.3), followed by cell morphogenesis (p= 2.07E-42), RNA polymerase II transcription factor activity (p= 1.85E-41), sequence-specific DNA binding (p=1.85E-41), regulatory region nucleic acid binding (p= 1.67E-27), regulation of cell growth (p= 2.05E-27), ameboidal-type cell migration (p= 2.69E-26), tissue homeostasis (p= 7.41E-23), developmental process involved in reproduction (p= 2.14E-22), DNA binding (p= 3.02E-21) and steroid hormone receptor activity (p= 2.24E-19).

A list of all significant overrepresented terms is provided in Appendix Table B3 with bird's-eye view presented in Figure 4.3 where all the significant terms can be found under the respective broad categories. Total number of genes associated in each term and their percentage is provided in Appendix B5.

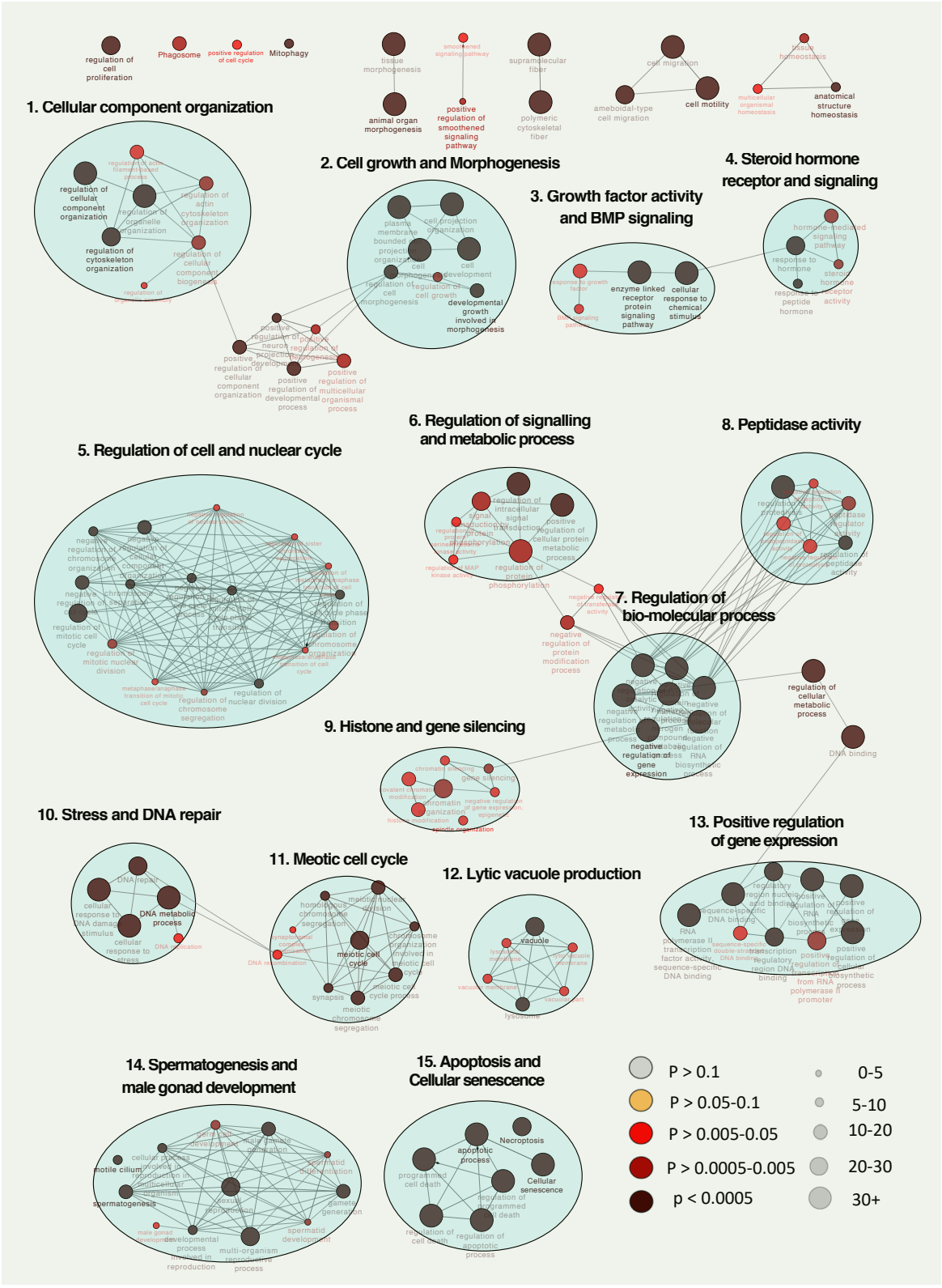


Fig 4.3 Go enrichment analysis of DEGs, summarising the distribution of GO terms, their significance (colour intensity scale provided) and the number of mapped genes in each category. Note: Terms with p > 0.05 were discarded. Node size represents the number of mapped genes associated in each category and edge represents the overlap of the genes between the two terms.

4.4.4.2 KEGG analysis

Similarly, when pathway analysis was performed using KEGG database; FoxO signalling (p=0.02), Progesterone mediated oocyte maturation (p=0.02), NOD-like signalling (p=0.02), MAPK signalling (p=0.02), mitophagy (p=0.03) and GnRH signalling (p=0.03) were additionally enriched. An overview of the KEGG pathway analysis is provided in Figure 4.4.

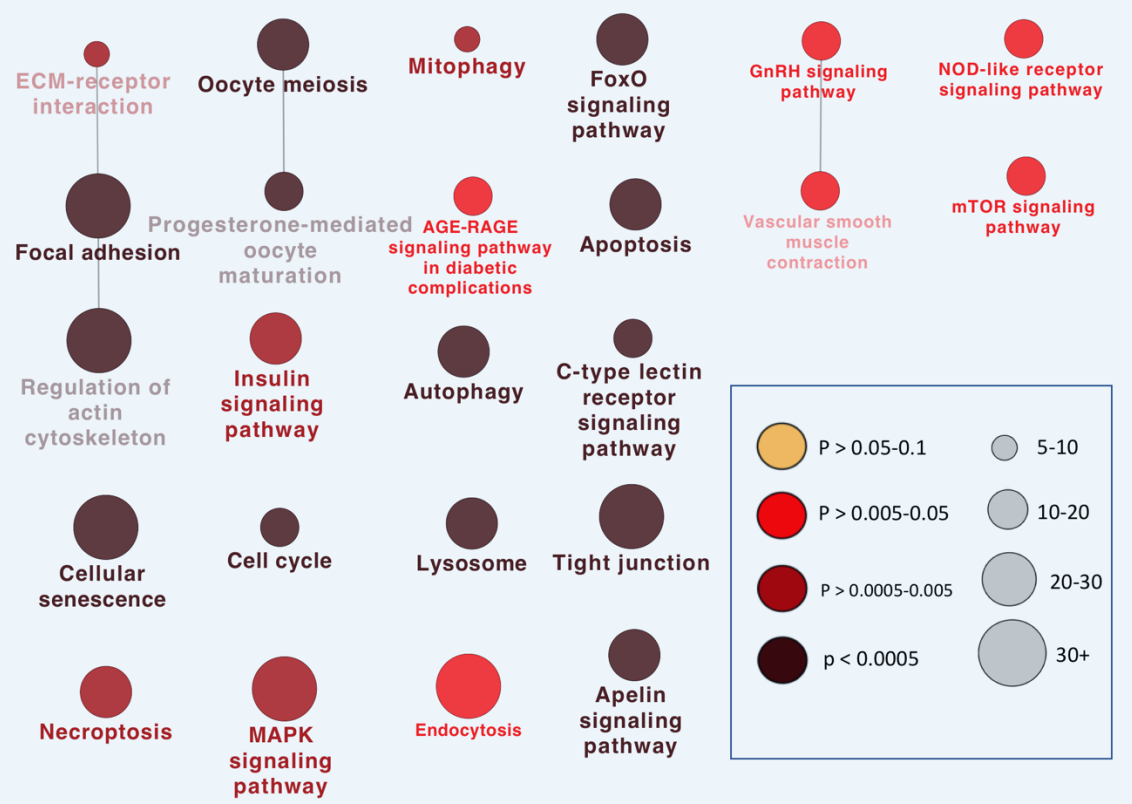


Fig 4.4 Summary of KEGG pathways affected (P <0.05?) in JGC testis. Node sizes and colour intensity correspond to number of genes and level of significance respectively.

4.4.5 Functional annotation and characterization of terms with association to morphology of JGC

Not surprisingly, pathway analysis of DEGs using GO and KEGG database revealed that several of the pathways directly associated with morphological changes observed in JGC tissue were significantly altered. Enriched terms associated with morphological (Chapter 2) and histopathological (Chapter 2 and3) observation are described below-

4.4.5.1 Cellular component organization

Consistent with loss of cellular integrity of the testis (chapter 2), the key sub-pathways affected were regulation of cytoskeleton (GO:0051493), organelle (GO:0033043), cellular component (GO:0051128) and actin (GO:0032970, GO:0032956). A total of 108 genes were found associated with the cellular component with highest fold changes observed in *apoeb* (5.6) followed by *anapc11*(-3.6), *ptk2ba* (3.6), *hormad1* (-3.5), *socs2* (3.4), *caprin2l* (3.3), *htra1b* (3.0), *rsg1* (-2.9), *triobpb* (2.7) and *cdc26* (-2.4) genes. Of those associated with cytoskeleton organisation 35 genes were affected and high fold changes were observed with *capn2l* (3.3), *gsnb* (2.7), *baiap2a* (2.5) genes that were all upregulated. Similarly, actin pathways were enriched with 20 genes associated with it. Interestingly, most of the actin genes were upregulated in JGC condition with the exception of *rhoab*, *spire1b*, *sptbn1*. Similarly, actin regulation was enriched in KEGG pathway as well (KEGG:04810) (Figure 4.5).

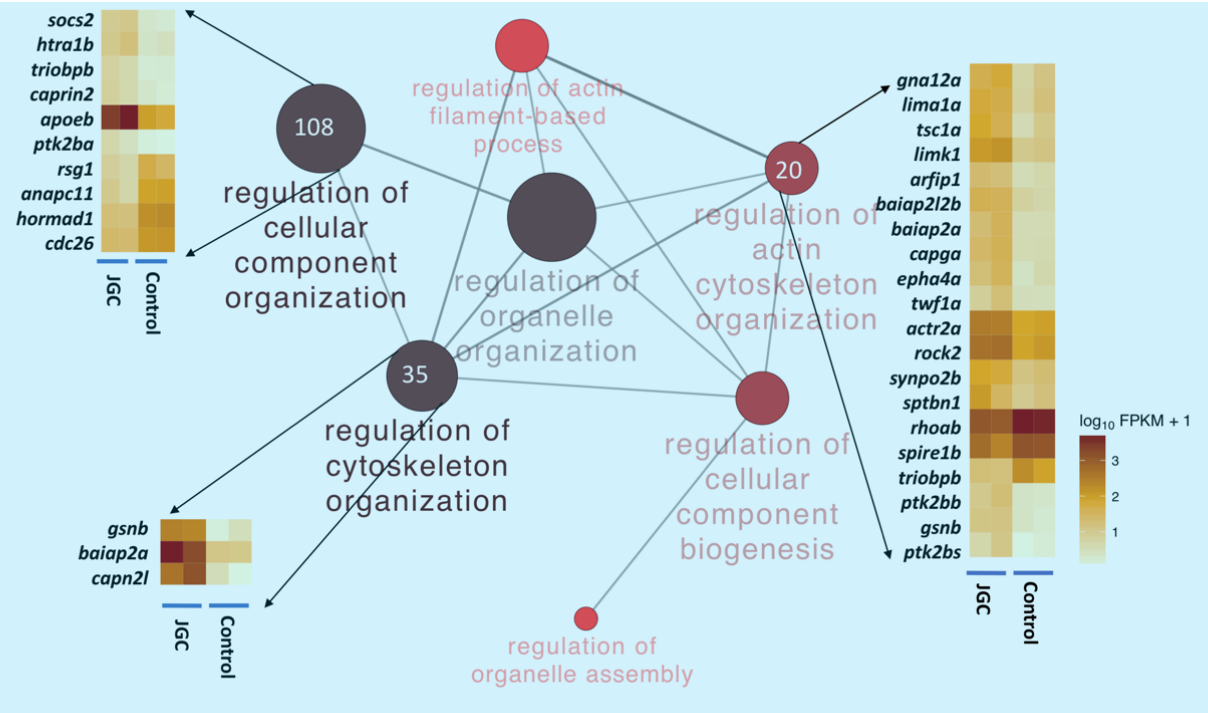


Fig 4.5 A summary of the genes with high fold changes associated with cellular component, cytoskeleton and actin regulation pathways. Note: Most of the genes in actin regulation were upregulated in JGC. Total number and heatmaps of select genes within the key nodes are inset.

4.4.5.2 Regulation of cell growth and morphogenesis

Regulation of cell growth and morphogenesis pathways that typically regulate organ formation were affected in JGC tissue. Among the 109 genes involved with animal organ morphogenesis (GO:0009887); *cyp26a1* (3.2), *cx43* (3.1) and *cxcl12a* (3.0) exhibited the highest (in descending order) upregulation in JGC whereas *pitx2* (-4.7), *foxj1a* (-4.4), *dnaaf1* (-4.0) had the highest downregulation. Interestingly, among 17 genes associated with regulation of cell growth; *apoeb* (5.6), *socs2* (3.4), *cxcl12a* (3.0) and *htra1b* (3.0) had the highest fold changes with all upregulated in JGC. A heatmap of the affected genes in tissue morphogenesis and regulation of cell growth pathways is provided in Figure 4.6.

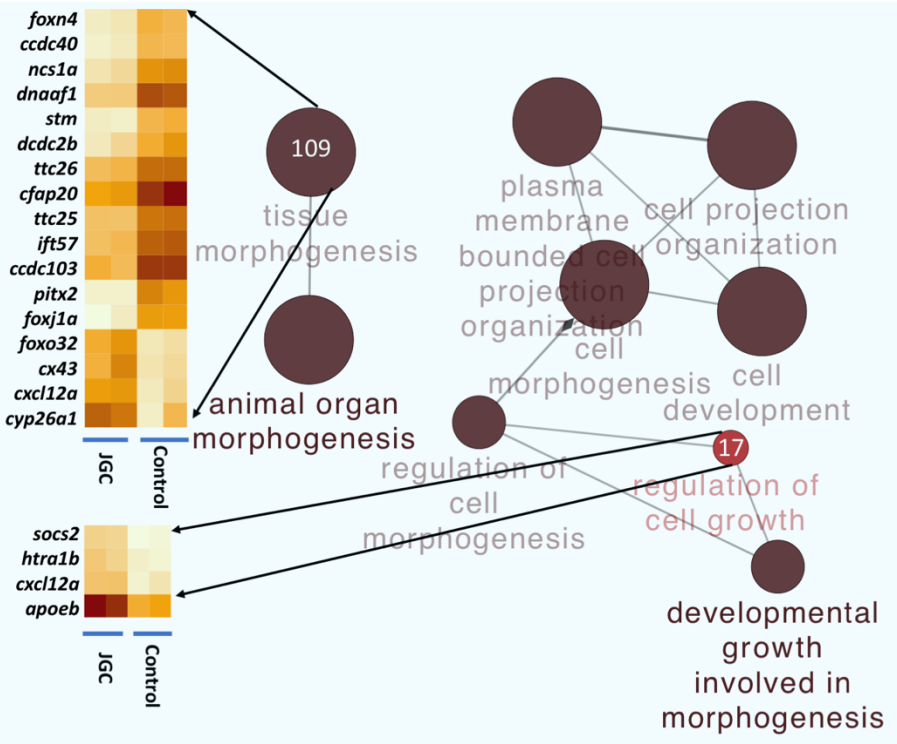


Fig 4.6 Expression of select genes (heat map) with high fold changes in morphogenesis and cell growth pathway. Heatmap of the key genes are presented with the corresponding nodes.

4.4.5.3 Growth factor and BMP signalling

Enrichment analysis and heat map of differentially expressed genes in the growth factor and BMP signalling pathway are presented in Figure 4.7. Specifically, response to growth factor (GO:0070848), BMP signalling (GO:0030509), response to hormone (GO:0009725), peptide hormone (GO:0043434), steroid hormone receptor activity (GO:0003707) and hormone mediated signalling pathway (GO:0009755) were affected by JGC condition. Most of the genes involved in growth factor pathways except *dynlrb1*, *rlim* and *skor1b* gene were upregulated. Similarly, complete upregulation of all the associated genes were observed for response to peptide hormone and steroid hormone receptor activity pathways (Figure 4.7).

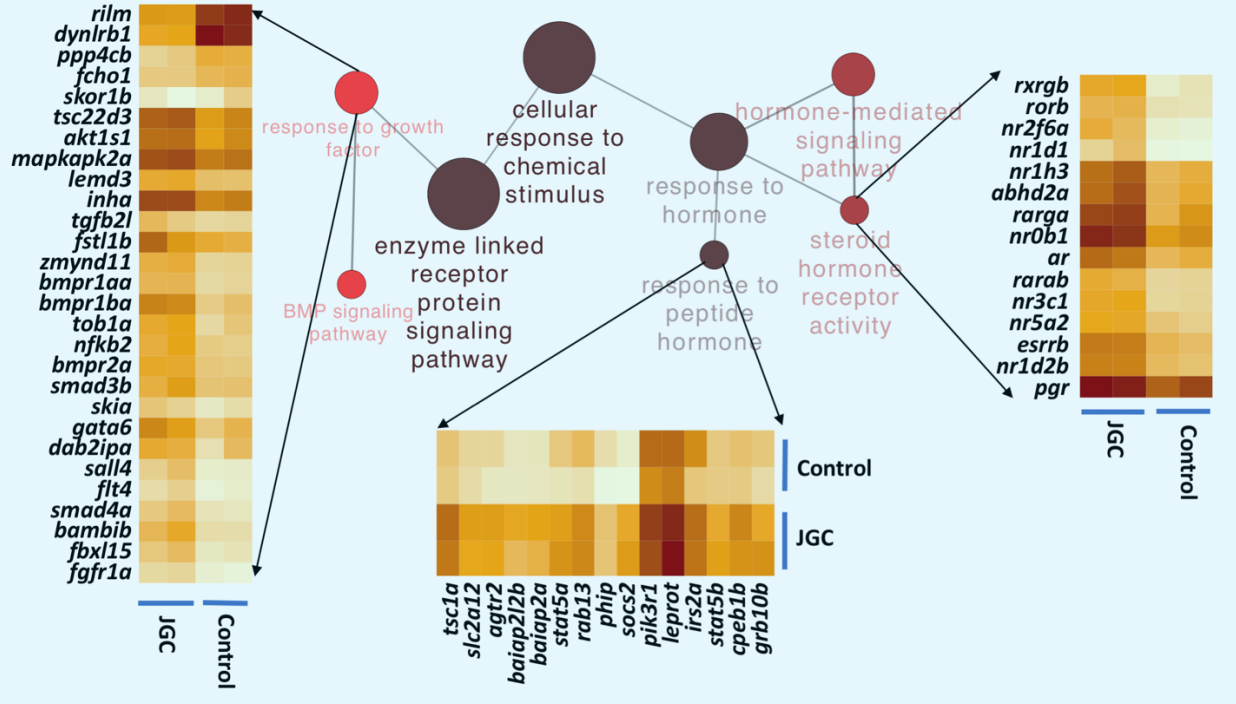


Fig 4.7 A summary of the genes involved in growth factor, steroid hormone receptor and peptide hormone pathways. Heatmap of the associated genes are presented with key nodes.

4.4.5.4 Regulation of cell proliferation, mitotic and meiotic cell cycle

A total of 37 genes involved in regulation of cell proliferation (GO:0042127) had altered expression pattern (Fig 4.8) with high upregulation of genes such as *cxcl12a*, *dner*, *fgfr1a*, *fynb*, *jun*, *ndrg1a*, *pdgfd*, *ptk2ba*, *sla1*, *tbx1* and *tob1a* and downregulation of *tfap2d* and *vegfbb* in JGC tissue. Interestingly, most genes in mitotic cell cycle (GO:0007346) pathway were downregulated, with a few exceptions including *dusp3*, *swap70b*, *gnl2* (Figure 4.8). In contrast, a complete downregulation of all the genes involved in meiotic cell cycle (GO:1903046) was observed (Figure 4.8, heatmap provided).

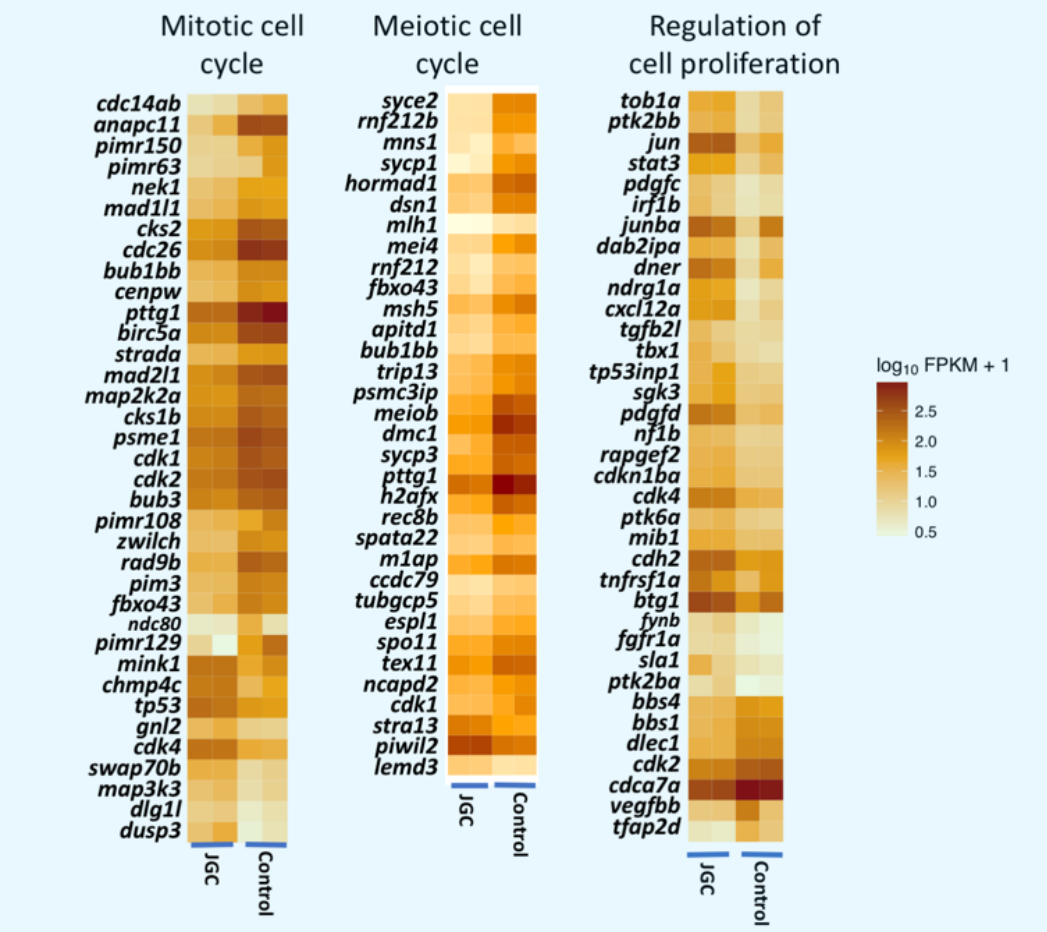


Fig 4.8 Differential expression of the genes involved in cell division (mitosis and meiosis) and proliferation. Note the downregulation of the genes involved in both mitotic and meiotic cell cycles, with the exception of notable few in mitotic pathway.

4.4.5.5 Regulation of proteolysis and lytic vacuole production

An irregular expression pattern of genes was found in proteolysis (GO:0030162) and lytic vacuoles production (GO:0005773) pathways. While most of the genes were found upregulated, highest fold changes were observed for *abtb2a* (2.8), *fbln1* (2.7) and *c4* (2.4). However, *rnf14* (-4.8), *bcl2l1*(-3.3) and *muc5.1* (-2.7) were highly downregulated in JGC (Figure 4.9).

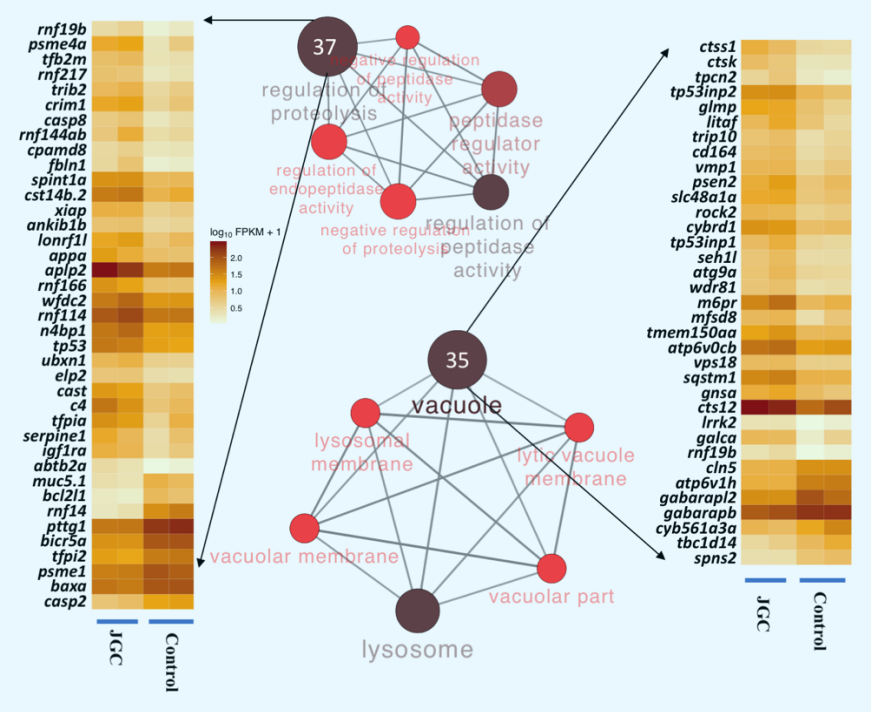


Fig 4.9 Heatmap of the genes involved in proteolysis and vacuole production.

4.4.5.6 Apoptosis and cellular senescence

Cellular senescence (KEGG:04218) and apoptosis (GO:0006915), the ultimate physiological process that are involved in regulation of cell proliferation and death were affected in JGC tissue. In apoptotic process, *apoeb*, *boka*, *dnase2*, *ptk2ba* were highly upregulated in JGC whereas *faima*, *ift57*, *pimr129* were found downregulated (Figure 4.10). Among 40 genes involved in cellular senescence 30 genes were found up upregulated with highest fold changes associated with *capn2l* (3.3). A heatmap of the salient genes involved in cellular senescence is provided at Figure 4.10.

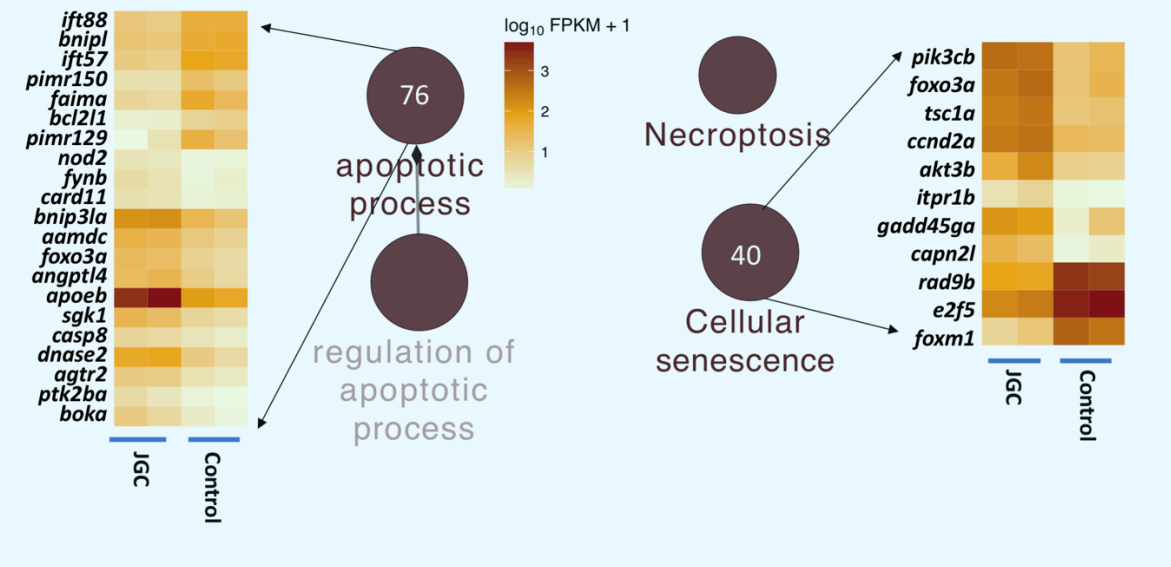


Fig 4.10 Heatmap of the genes involved in apoptosis and cellular senescence. Note: Necroptosis was also enriched in the JGC condition.

4.4.5.7 Spermatogenesis and male gonad development

As expected, spermatogenesis and male gonad development pathways that generally determine the reproductive health of an individual were significantly affected in JGC tissue. At least 22 DEGs were affected in spermatogenesis pathway, with most of them down regulated. Among the downregulated genes highest fold changes were observed with *ropn1l* (-5.1), *klhl10a* (-4.9), *meig1* (-4.2), *dnaaf1* (-4.0), *hormad1* (-3.5), *mei4* (-2.9), *tsga10* (-2.9) and *cfap157* (-2.8). However, an upregulation of *psme4a* was observed in spermatogenesis. Surprisingly, five genes involved in male gonad development including *dmart1* were upregulated in the JGC condition, with the exception of *klhl26* (-4.86 fold) which was downregulated (Figure 4.11, heatmap provided).

4.4.5.8 MAPK and GnRH signalling

The MAPK signalling pathway (KEGG:04010), that is typically associated with tumorigenesis was significantly altered, with about 52 genes affected. While most of the genes in GnRH signalling pathway were upregulated, highest fold changes were observed

with *jun* (3.0), *cacna1fb* (2.9), *camk2b* (2.7), *adcy6b* (2.6), *itpr1b* (2.3), *mmp14a* (2.2), *mmp2* (2.2) and *adcy9* (2.0) (Figure 4.11)

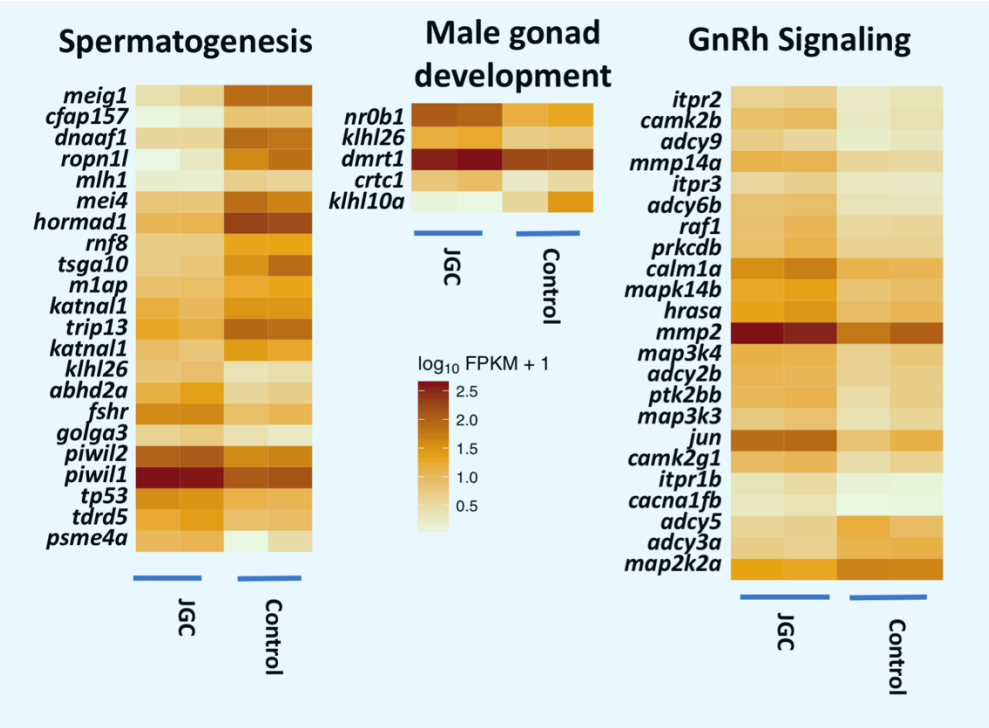


Fig 4.11 Differential expression of the genes involved in spermatogenesis, male gonad development and GnRH signalling pathways.

4.4.5.9 Other significantly altered pathways

In concurrence with general structural degeneration of JGC carp testis, most of the downstream pathways were affected— most noticeable of which were the downregulation of genes involved in both calcium binding and histone metabolism/signalling pathways in JGC condition, with the exception of those involved in histone 5 metabolism. (Figure 4.12).

Interestingly, however, the collagen pathway was significantly upregulated in the JGC condition with the exception of *col17a1b*. (Figure 4.12)

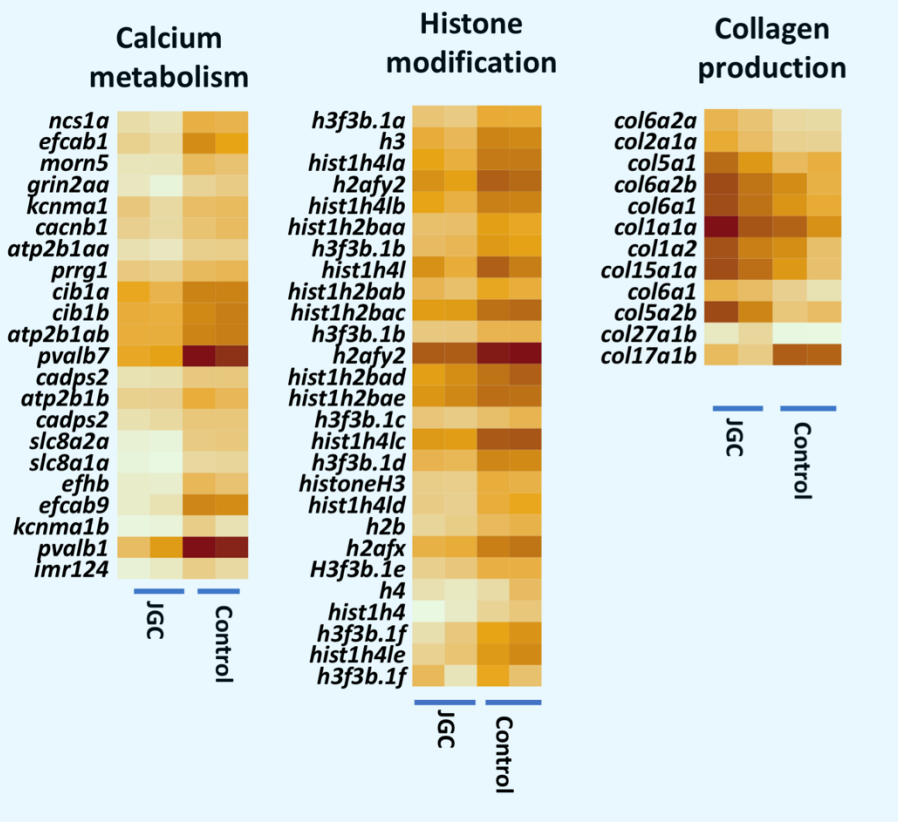


Fig 4.12: Heatmap of the genes involved in calcium metabolism, histone modification and collagen production. Note: the genes involved in calcium and histone pathways were mostly downregulated while those in collagen metabolism were upregulated in JGC testis.

4.5 Discussion

A sequential and tightly controlled gene expression is key for structural and functional integrity of tissues. Transcriptome being a complete collection of all expressed RNA at any given time provides the best diagnosis of this integrity. With scarcity of genomic resources for most non-model species, next generation sequencing (NGS) offers the greatest hope for unravelling the molecular mechanism (Ji et al., 2012) of rare conditions such the one described here. Using NGS technology, the transcriptome profile of JGC carp with a unique gonad deformity that shows signs of early proliferation of Sertoli cells, loss of spermatogenesis, degeneration of germ cells, followed by complete loss of an organ function (detailed in chapter 2) is presented here. Analysis in JGC testis revealed a severe irregularity in gene expression in JGC testis.

Of the clean reads, about ~24.1% were not mapped possibly because of the uncovered regions of the reference genome, sequencing errors, anchor region spliced alignment, deletion of more than 4 bp or a possible difference in the genome of Tasmanian carp and that of the reference genome. Among the DEGs at least 17% of the genes were completely uncharacterized at the time of this analyses. A confounding factor also was the assignment of multiple DEGs to the same gene name. This despite significant effort made to overcome the unannotated genome of common carp by manual characterization of the DEGs and addressing duplicated DEGs in isomers when possible. However, this can be explained by the fact that common carp is a pseudo tetraploid organism (Xu et al., 2014) with many duplicated genes, resulting in multiple DEGs with the same name. This highlights the need for finer delineation of duplicated paralogs, their distinct nomenclature and revised annotation of the carp genome.

While the incomplete reference genome and its relatively poor annotation (compared to human or Zebrafish) was a limitation, this was largely overcome by not exclusively limiting the mapping to annotated parts of the genome. Instead unannotated genes were addressed as expressed contigs.

4.5.1 Differentially expressed candidate genes

Primary interest of this study was to detect expression signatures associated with JGC condition, with particular emphasis on those corresponding to morphological and histological observations of fluid accumulation, cellular proliferation, tissue degeneration, and loss/death of germ cells (chapter 2) with a possibility of identifying cause of the condition. Although many genes (7,129) were found to be differentially expressed at statistically significant level, most of these are likely to be a consequence (effect) of the condition. For example, as the complete morphology of the testis is disrupted with the condition, a large transcriptomic change is expected. The hierarchical

ranking based on fold changes identified 40 genes as prime candidates influencing the disease condition. Overall these 40 genes fall into two general categories—those that are 1) uncharacterised and 2) already characterised.

4.5.1.1 Aberrantly expressed uncharacterized locus/RNA

Interestingly, more than half of the highest differentially expressed genes (26 out of 40) are novel and appeared undescribed in any other species before. Homology search (BLAST) of the top 26 uncharacterized genes indicated that five ncRNAs are included in this group, however, appears novel to date. However, such discovery of novel genes are common in RNAseq application (Jabbari et al., 2012) as it is a superior technology that do not limited by the existing knowledge.

It is quite possible that these 26 uncharacterized genes (either as a coding or non-coding) are directly regulating critical functions of gonad, spermatogenesis or cancer. However, careful investigation of those locus indicated that many of the regions are unusually long and contain multiple ORFs. Future studies evaluating these 26 genes could reveal their functions in testicular tissue and JGC condition. Additionally, future study should also cover any defect of these selected genes due to point mutation, insertion or deletion that may be responsible for the JGC condition. Moreover, an in-silico characterization detailing the protein domain and tissue specific expression could also be helpful characterising those genes.

4.5.1.2 Aberrantly expressed characterized genes

Within the characterized genes, a significant downregulation of two isomers of *spaca9* gene was observed in JGC testis. *SPACA* (sperm acrosome-associated protein) proteins are generally known to be testis specific and localized in acrosomal matrix (Korfanty et al., 2012). Much of the function of the proteins belonging to *SPACA* gene family including *SPACA9* is still speculative (Korfanty et al., 2012). However, in mouse some

SPACA proteins were reported to be regulated by 'Y' chromosome (Bhattacharya et al., 2013), therefore downregulation of *spaca9* in this study combined with male-specificity of JGC condition (Chapter 2) indicates that the JGC condition could be Y linked, however, this needs confirmation. Similarly, *tex36* that was found downregulated in this study is a common gene expressed in testis (Katsanis et al., 2017). Downregulation of *tex36* has been previously linked with male infertility in cattleyak (Cai et al., 2017). It is therefore likely that either or both of *tex36* and *spaca9* could play key role in the development of JGC testis and are prime candidates for future studies to understand the observed sterility of JGC fish and elucidating their function in testis development and sperm production in animals.

The observed downregulation of *ropn1l* could be associated with reduced spermatozoa capacitation observed in JGC fish (chapter 3). For example, recent studies indicates that *ropn1l* has a role in phosphorylation of fibrous sheath proteins in mouse spermatozoa and aid sperm movement (Zhang et al., 2016) and loss of this protein results in defective murine sperm motility (Fiedler et al., 2013). As, *ropn1l* is recently considered as a target gene to treat male infertility (Zhang et al., 2016), the JGC carp could serve as a good model to study *ropn1* function as well test associated fertility therapies, complimenting the existing mouse model.

It is generally known that the sperm DNA is compact and tightly packed inside the spermatozoa and nucleo-histone plays a crucial role in packing and decondensing the DNA (Gatewood et al., 1987). As the content of spermatozoa sharply decrease in JGC testis, the significant downregulation of the histone genes could be a generic response of this effect. Similar generic response could be attributed *spata4* a testis specific gene that maintains key physiological function in germ cell (Jiang et al., 2015).

While most of the top associated genes were downregulated, *pvalb1* and *scg2a* (secretogranin-2-like) were highly upregulated in JGC tissue. Much of the functions of *pvalb1* is not known to date, although polymorphism of this gene has been linked to increased growth of fish (Xu et al., 2006). Upregulation of *scg2a* gene in JGC condition is somewhat intriguing. The function of this gene is barely known, with the exception of an implied role in skin pigmentation of the fish (Sivka et al., 2013). However, no pigmentation of the JGC testis has been observed till today. Of the downregulated genes, the role of *odf3b*, a gene with highest fold changes could be generic response of lacking spermatozoa as *odf3b* has been described as a sperm tail protein (Marra and Wingert, 2016).

4.5.2 Pathways affected in JGC tissue

Despite the incomplete annotation of the *C. carpio* genome, Go and KEGG analyses, recognised key pathways that reflect the cellular changes observed (chapter 2 and 3) in the JGC condition. Salient features corresponding to these pathways are discussed below.

4.5.2.1 Cellular component organisation was impaired in JGC

Consistent with the structural degeneration of the tissue, genes involved in cellular component organisation were critically impacted in JGC. As many as 109 genes involved in component organisation were impacted with majority downregulated notable among which were downregulation of *rsg1*, *anapc11*, *hormad1* and *cdc26*. Regular expression of these genes is required for proper structural integrity and function of any given tissue. For example, *rsg1*, a small GTPase (guanosine triphosphatase) is involved in cilia formation, hedgehog signalling and microtubules elongation (Agbu et al., 2018), all essential for intracellular communication (Bangs and Anderson, 2017). In contrast, the upregulation of a subset of genes such as *anapc11* and *hormad1* that are associated with carcinogenesis (Carlin et al., 2015; Chan et al., 2001) suggest a possible, uncontrolled

proliferation of some if not all cell types. Especially *hormad1* is commonly known to be enriched in cancerous-testis (Yao et al., 2014). This observation concurs with the observed morphological changes in stage 1 JGC, where the Sertoli cells were significantly increased in number (chapter2). Similarly, all the genes involved in actin filament organization process were found upregulated indicating an increased filament assembly, required to support the uncontrolled proliferation of cells in the JGC testis. The Sertoli cells and seminiferous epithelium are actin rich (Vogl, 1990) and upregulation of actin genes could explain the anatomical observation (chapter 2) of thick seminiferous tubules due to the over proliferation of Sertoli cells and the supporting epithelia (Chapter 2).

Similarly, *socs2* is considered as a vital gene for cellular function, regularly expressed in many organ including testis (Dey et al., 1998). However, *socs2* is also considered as a candidate gene for suppressor of cytokine signalling family (Letellier and Haan, 2016) highly expressed in breast (Farabegoli et al., 2005; Sutherland et al., 2004), prostate (Zhu et al., 2013) and pulmonary (Wikman et al., 2002) cancers. Additionally, *socs2* is known to regulate growth hormone production (Letellier and Haan, 2016). For example studies in vitro have determined that low levels of *socs2* led a reduced growth hormone signalling while, increased growth hormone signalling was observed with higher levels (Favre et al., 1999). Collectively, upregulation of *socs2* supporting the cancerous nature JGC while potentially influencing the growth rate of JGC fish (chapter 2). This also supports the hormonal imbalance observed in JGC fish (Chapter 3). Collectively, these results demonstrate that the observed cellular component perturbations parallel the anatomical observation of gross testicular degeneration as well as increased proliferation of a subset of cells (e.g Sertoli)/tissue in JGC tissue.

4.5.2.2 Regulation of morphogenesis and cell growth

Morphogenesis is the biological process by which multicellular organism controls the organisation/distribution of cells in an orderly fashion leading to development of a fully functional organ. Enrichment analysis of DEGs revealed a significant number of genes in the morphogenesis pathway were affected in JGC testicular tissue. Significantly, most of the genes related to morphogenesis were found downregulated in JGC condition indicating premature developmental arrest of many cell types. Among those downregulated genes, high changes were observed with *dnaaf1*, *foxj1a*, *ccdc103*, *mpp5a*, *ncs1a*, *pitx2* and *stm*. In particular, most of the downregulated genes are specific to formation to motile cilia. For example, it is generally known that transcriptional factor for *foxj1* are the master regulator of motile ciliogenic process (Yu et al., 2008), therefore *foxj1a* is required for proper development and organ homeostasis (Hellman et al., 2010). Specifically, mutation and absence of *dnaaf1* is linked to disrupt cilia formation and can increase the risk (94%) of testicular germ cell tumour (Litchfield et al., 2016). Similarly, *ccdc103*, *ttc26*, *ttc39c* are important factor which are essential for motile cilia development (Ishikawa et al., 2014; Panizzi et al., 2012; Xu et al., 2015b). Collectively, downregulation of all the genes involved in motile cilium, including their regulators were observed in JGC testis which was also reflected in the enrichment result (motile cilium: GO:0031514). Cilia play important functions of physiological and developmental process. Lack of functional cilia is the leading cause of several disease called ciliopathies. While cilia and flagella is a key component of spermatozoa and testes (Pasek et al., 2016), lack of this pathway may lead to male sterility that has been observed in JGC fish (Chapter 2 and 3). Significantly, loss/disfunction of motile cilium is recently recognised as important indicator for multiple cancer types (Wong et al., 2009; Yuan et al., 2010), therefore the observed suppression of these genes in JGC testis may suggest a dysregulation of morphogenesis possibly from a cancerous etiology.

A set of genes related to cell growth (e.g. *cx43*, *arnt2*, *cxcl12a*, *cyp26a1*, *fbxo32* etc.) exhibited an upregulation pattern. Specifically, *cx43* is known to control the proliferation of Sertoli cells (Gilleron et al., 2009), and therefore is in agreement with the observed Sertoli cell proliferation (chapter 2). Moreover, *cx43* is linked with testicular pathogenesis and considered as cancer biomarker (Chevallier et al., 2013). Although, the exact roles of *fbox32* and *arnt2* is not clear they have also been linked to carcinoma and cell growth (Kimura et al., 2016; Yuan et al., 2018). Their upregulation further supports the biological observation of Sertoli cell proliferation in JGC tissue.

Within the cell growth pathway, *pitx2* controls multiple functions of gonad including maintaining shape and size of the testis (Rodríguez-León et al., 2008). It's downregulation could explain the observed (chapter 2) loss of morphological integrity of the JGC testis.

4.5.2.3 Growth related activity

As presented in chapter 3, growth analyses on a large number of JGC fish has demonstrated an accelerated growth rate of the JGC fish compared to their unaffected cohorts. In agreement with this, the pathway analysis revealed enrichment of growth related pathways including response to peptide hormone and steroid hormone receptor activity pathways. Especially, genes involved in these pathways were mostly upregulated signifying their increased activity in JGC tissue. For example, members of nuclear receptor (*NR*) genes upregulated in JGC fish belonged to the circadian clock regulator pathway. Disruption of circadian clock not only promotes metabolic rates and feeding but also contribute obesity and cancers (Evans and Davidson, 2013; Guerrero-Vargas et al., 2017). In particular, study of JGC transcriptome indicates high upregulation of *nr1d1*, *nr1d2b*, *nr2f6a* including other *NR* genes (Figure 4.7) which are essential component of circadian clock. For example, study in humans and zebrafish proved the *nr1d1* have

multiple functions in autophagy, nutritional signalling and obesity (Huang et al., 2016). Also, *nr1d2b* can interact with steroid hormone, gonad and reproduction (Zucchi et al., 2013). Therefore, observed enhanced growth of JGC fish could be a result of circadian clock dysregulation, an aspect that warrants further investigation.

4.5.2.4 Regulation of mitotic cycle, meiotic cycle and cell proliferation

As presented in chapter 2, histological observations of JGC testis revealed an initial proliferation of Sertoli cell (somatic cells) with concurrent loss of spermatogenesis (germ cell). Routine cell division in testis can be categorised into somatic cell division and germ cell division. The rapid cycle of cell division to produce germ cell is a combination of mitotic and meiotic cell cycle, while, the somatic cell in testis maintain their population using only mitotic division (Schulz et al., 2010). Most of the genes associated with mitotic and meiotic cell cycle were downregulated with high fold changes signifying the arrest of both cell cycles. This explains the loss of spermatogenesis in the condition. In mitotic cell division pathway, high downregulation of *pimr129*, *pimr108*, *pimr150* and *pimr63* was observed, this can be largely attributed to complete arrest of spermatogenesis, that otherwise contribute to bulk of the cell proliferation in a healthy and mature testis in a peak reproductive cycle. While most of the genes involved in mitosis were downregulated, *dusp3* (dual-specificity phosphatase) was upregulated, suggesting that a subpopulation of the testicular cells were indeed proliferating higher than in the control tissue. Though *dusp3* were attributed to regulate the cell division and proliferation, the exact function of the gene is not known; however, it is commonly known to be overexpressed in cancerous cells (Panico and Forti, 2013).

Similar to mitotic cell division, all the genes involved in meiotic cell cycle also exhibited a complete downregulation supporting the morphological observation of meiotic arrest and this was also reflected in pathway enrichment analysis. Major downregulation of

dmc1, *dsn1*, *hormad1*, *rnf212b*, *syce2*, *sycp1* and *sycp3* observed. Specifically, *dmc1* is a recombinase, essential for synapsis during meiosis I to produce healthy spermatozoa (Chen et al., 2016). Downregulation of the gene may thus explain the higher sperm abnormality associated with JGC (chapter 3). Similarly, *hormad1* is essential for mitosis and meiosis (Fukuda et al., 2010) and is highly expressed in developing gonad (Pangas et al., 2004; Shin et al., 2010). Downregulation of this gene therefore indicates a gross dysfunction of the JGC testis. In some instances, *hormad1* is defined as an oncogene or cancer testis antigen that may also overexpress in gastric (Aung et al., 2006), breast (Adélaïde et al., 2007) and ovarian (Shahzad et al., 2013) cancers as in head and neck squamous carcinoma (Carlin et al., 2015). Interestingly, in-situ experiments targeting *hormad1* with siRNA-DOPC discovered the reduction of cancer growth and angiogenesis (Shahzad et al., 2013). Additionally, complete knockdown of *hormad1* in-vitro revealed the reduction of cancer cell line through induction of apoptosis (Carlin et al., 2015). Therefore, its downregulation in JGC testis is contradictory to the cancerous etiology hypothesis. Nonetheless, this can be explained by gross loss of spermatogenesis in JGC, making its otherwise elevated expression in uncontrolled (cancerous) proliferation of Sertoli cells observed (chapter 2). It is also possible that the observed mass loss (cell death) of all testicular cell may be mediated by the downregulation of *hormad1* that needs further investigation. In this regard, JGC common carp could potentially be a unique model for studying apoptosis of cancer cells with hormad1 as a key marker of the process.

Although, mitosis and meiosis pathways were down regulated in JGC tissue, genes involved in cell proliferation exhibited a high upregulation pattern. It is therefore quite possible that upregulation of these genes may be linked to the simultaneous Sertoli cell proliferation observed (chapter 2). In fact, genes like *cxcl12* and *ptk2b* are not only highly expressed in Sertoli cells (Beverdam et al., 2010; Gilbert et al., 2009) but also *cxcl12* is considered as a marker for stage I non-seminoma germ cell tumour (Gilbert et al., 2009).

Similarly, genes like *ndrg1* are known to promote cell proliferation in a subset of tumours (Byun et al., 2018). Clear relationship between many genes related to cell proliferation in animals at large is not yet clear. However, this aberrant expression of genes related to proliferation may be a result of feedback regulation of cancer cycle or uncontrolled proliferation of Sertoli cell in the initial stage. Indeed, recent results are suggesting the capability of feedback loop to dysregulate proliferative signals (Hanahan and Weinberg, 2011).

4.5.2.5 Regulation of proteolysis and vacuole production

Differential expression of the JGC transcript indicated a significant number of genes are affected from proteolysis and vacuole production pathway. However, exact roles of the genes involved in this pathway are as yet unclear. It is conceivable that proteolysis in JGC tissue contributes to collapse of the testicular tissue producing fluid filled vacuoles that was observed in gross anatomy of the testis (Chapter 2). However, proteolysis and vacuole production in gonad tissue is somewhat unique and never been described. In this regard the JGC fish could serve as unique stem to study and understand both the process.

4.5.2.6 Apoptosis and autophagy

It is generally believed that apoptosis or type I programmed cell death serve as a natural barrier of cancer development or progression (Adams and Cory, 2007; Hanahan and Weinberg, 2011). Although, malignant cancer cells are well-known for their ability to evade apoptosis or autophagy, somehow JGC cells in testicular tissue seems to be affected vigorously by this process—76 genes in apoptotic pathway were impacted. Apoptosis is performed by intracellular cysteine proteases namely caspases (Adams, 2003), which largely remain inactivated in normal state of cell, therefore requires specialised activation for its function. There are two independent major activation pathways- 1) 'stress mediated' pathway and 2) 'death receptor mediated' pathway. However, the stress

pathway, which is also called ‘intrinsic’ or ‘mitochondrial’ pathway is highly regulated by the *bcl2* family. In this study *bcl2l1* was found to be highly downregulated in JGC tissue. Although, the exact mechanism of the *bcl2* in apoptosis is still a mystery, it is generally believed that members of the *bcl2* are considered as apoptotic inhibitor as they bind to apoptotic triggering proteins (*bax* and *bak*) and inhibit their activity (Adams, 2003). Downregulation of this gene indicates a stress mediated apoptosis in JGC tissue. Stress may be triggered in response to a wide range of processes, including physiological stress of tumorigenic or oncogenic process (Adams and Cory, 2007). Typically, stresses from signalling imbalance, DNA damage due to hyperproliferation can result in apoptotic induction (Hanahan and Weinberg, 2011). However, it is known that, stress pathway activates *casp9*, that was not differentially expressed in JGC tissue. In contrast, *casp8* a member of ‘death receptor’ pathway was upregulated (Adams and Cory, 2007). While it is tempting to suggest that the observed cell loss in JGC is triggered by death receptor mediated pathway, it is entirely possible that both pathways are contributing factors.

Pathway analysis using KEGG database suggest enrichment of autophagy (KEGG:04140), which is known as type II programmed cell death mainly mediated by the lysosomal system (Gozuacik and Kimchi, 2004). In general, autophagy is governed by a set of evolutionary conserved genes namely *atg* genes (Meijer and Codogno, 2004; Yorimitsu and Klionsky, 2005). Although, over 30 orthologs have been identified in yeast only few (*atg*1, 3-10,12 and 16) have been detected in mammals (Thorburn, 2008). Sometimes, in mammalian tissue *ulk1* (an ortholog of *atg1*) can also control autophagy (Chan et al., 2007). The induction of autophagy starts with the serine/threonine kinase (*atg1* gene) activity, followed by the nucleation of the vesicle mediated by *pik3*. The whole process is dependent on formation and expansion of autophagosome that requires cascades of events involving multiple proteins, namely *apg*, *ras*/*raf-1*, *atk*, *bnip3*, *dapk*; that finally results in successful autophagy (Gozuacik and Kimchi, 2004; Thorburn,

2008). Not surprisingly, transcriptome analysis of JGC revealed the upregulation of *atg*1, 4, 9 along with *ulk1* which indicates the activation of autophagy pathway. Unlike apoptosis, autophagy stimulates a number of other pathways; for example, atk signalling, growth factor signalling, MAPK signalling, proteolytic activity, small GTPase activity, inositol triphosphates, calcium signalling, mTOR signalling among others (Thorburn, 2008). In agreement with this, the transcriptome expression in JGC tissue revealed a complete upregulation of all these accessory pathways strongly suggesting involvement of autophagy in JGC tissue.

In addition to the two major modes of cell death —apoptosis (type I cell death) and autophagy (type II cell death)— the third type of cell death, namely necrosis/necroptosis (distinct but less regulated than the other two) was also enriched in JGC tissue. Although, different modes of cell death were thought to be independent (Tsujimoto and Shimizu, 2005), recent research review has indicated that these pathways are interlinked (including necrosis) and they work together to regulate cell death (Gozuacik and Kimchi, 2004; Scott et al., 2007; Thorburn, 2008). For example, steroid mediated Drosophila's autophagic cell death (Baehrecke, 2003) is controlled by the regulators (e.g. reaper, dronk and grim) of apoptosis (Lee et al., 2002), and inhibition of caspases is sufficient to inhibit the total autophagic process (Martin and Baehrecke, 2004). In this regard, the characteristics of cell death pathways observed in JGC supports the latest theories of cell death. So far, the models to understand cell death are limited to Drosophila, yeast and tumour cell lines. The study of cell death is quite unique and complex in nature, as this often requires induction of the pathways externally using stress (for example starvation, steroid treatment etc.) or chemotherapeutic agents. Such extraneously induced stress may result in biasing the cellular environment masking observations. In this context, the JGC condition may offer a perfect and naturally occurring model that could enhance our understanding of apoptosis, autophagy, necroptosis and their interactions.

4.5.2.7 Cellular Senescence

Cellular senescence can be defined as an irreversible condition of growth arrest, triggered by multiple molecular process e.g. DNA damage, telomere shortening, ageing, epigenetic depression etc. (Collado et al., 2007). As senescence is considered to supress cancer, any cancer cell needs to bypass this mechanism for its survival. Although, it was previously believed that cancer cells loose their ability to senescence, recent research is indicating that it is an intrinsic trait to all cells and can be induced at any time in the life cycle of cancer cells (Roninson, 2003). The expression signature of JGC testis initially shows signs of cancer including extreme proliferative signals associated with carcinogenic process but is also marked by activation of senescence pathways at later stages. One example to support this idea is the downregulation of *foxm1* observed in JGC tissue. *foxm1* is a member of transcription factor (Forkhead box superfamily) not only frequently expressed in proliferating tissue including a broad range of cancers (Teh, 2012) but also depletion of it can cause loss of long term cell proliferation ability (Alvarez-Fernández and Medema, 2013). Therefore, downregulation of this gene is a further indication of activation of cellular senescence.

4.5.2.8 Spermatogenesis and male gonad development

Reproduction is an essential biological process critical for producing offspring of an individual. The complex process of spermatogenesis can be divided into four broad stages in cyprinids (Rupik et al., 2011) with the process of testicular development and germ cell production strictly regulated by molecular mechanisms (Fan et al., 2012). The observation of spermatogenic arrest in the JGC tissue (chapter 2 & 3) therefore is likely reflected in the gene expression pattern in the condition. Consistent with this prediction, several genes associated with cilia/flagella assembly, testis development, sperm stability were highly under expressed in JGC testis. For example, *dnaaf1* gene is not only essential for morphogenesis and cell growth but also crucial for assembly and stability of cilia and

sperm flagella (Xu et al., 2015b) that was significantly downregulated. Moreover, deletion or absence of *dnaaf1* in testicular tissue could result in male infertility (Xu et al., 2015a). Similarly, *klhl10a* has been linked with spermatogenesis and known to be regularly expressed in fish testis (Lee et al., 2017), which was highly down regulated in JGC testis. Downregulation of *meig1*(Li et al., 2015), *tsga10* (Sha et al., 2018) and *ropn1l* (Fiedler et al., 2013) have been linked to the reduced fertility and poor sperm health as was also observed in JGC carp. Additionally, *mei4* is required for double strand break during meiosis (Kumar et al., 2015), which works in association with *hormad1* and *dmac1* (Kumar et al., 2015) that were also downregulated in JGC testis.

Collectively, this study highlighted downregulation of many crucial spermatogenic genes in JGC testis. As in every tissue, strict spatio-temporal expression of genes is important for structural and functional integrity of testicular tissue. Any disruption to these patterns can be result in loss of spermatogenesis as was observed in the JGC tissue. While likely, it is as yet unclear as to what master gene in the spermatogenetic pathway is triggering the testicular degeneration and testis dysfunctionality observed in this study.

4.5.2.9 Other important pathways

Apart from the known pathways related to JGC condition, a number of other pathways were also affected in JGC tissue. For example, upregulation of most of the genes in GnRH signalling and collagen pathways and downregulation of calcium pathway were observed. However, the involvement of this pathways in JGC production and regulation is not clear at this stage but will be covered in our future study particularly GNRH pathway. However, no relation to the immune system pathways/processes again confirms a non-pathological etiology of the condition.

4.6 Conclusion

Overall, this study represents 7,129 genes that were differentially expressed between JGC and normal testis that could be a future resource for the study of common carp reproductive biology, spermatogenesis, testis morphology and testicular non-germ cell cancer at a large. Especially, as the many of the top genes are not characterized to date, future follow up gene expression study could provide a unique chance to discover/characterise novel genes, including onco/proto-onco genes.

Expression signatures of JGC testis perturbed many genes and pathways essential for structural and functional integrity of the testis, as well as those involved in induction cancer and apoptosis. For example, *sall4*, a gene considered as a strong and robust diagnostic marker for testicular cancer (Cao et al., 2009) was strongly upregulated along with other cancer markers (described above) indicating the condition has cancerous element. Most importantly, this study has identified several contigs that may harbour master regulator of key pathways in testicular tissue, however systematic delineation of these and their role is warranted. As there is a knowledge gap on the mechanism for non-germ cell malignancy and testicular apoptosis, this study offers valuable data to understand non-germ cell cancers, apoptosis of the malignant cells and spermatogenesis and reproduction in a broader context.

The observations could also contribute to discovering novel genes for fish sterility. Sterility of fish is desirable for aquaculture, pest management and research. While efforts have been made to sterilise carp surgically (Patil et al., 2015) and chemically (Ali and Rao, 1989; Twohey et al., 2003) for specific applications, this naturally occurring sterility could open the door for a molecular approach for sterilizing fish, whilst exploiting the enhanced growth performance observed (chapter 3).

Chapter 5: General Discussion

Lake Sorell

Studies of gonadal tissue abnormalities provide an opportunity to understand their functions (Barseghyan et al., 2018), physiology (Down et al., 1988; Litchfield et al., 2015; Odell and Swerdloff, 1978), pathogenicity (Chapman et al., 1981; Cools et al., 2006; Savage and Lowe, 1990) and impact of pollution (Down and Leatherland, 1989; Guillette Jr et al., 1994). Fish are considered important species for studying gonadal abnormality and related endocrine response due to their direct contact with the surrounding environment (Arcand-Hoy and Benson, 1998). In particular, fish reproductive capability respond very adversely due to environmental deterioration (Bittner, 2009; Dickman and Steele, 1986) and genetic predisposition (Down, 1984). While, the increasing awareness of environmental pollution and anthropogenic activity have attracted research on detrimental effects of chemicals on reproductive (Hecker et al., 2006; Jobling et al., 1998) and endocrine systems (Jobling and Tyler, 2003; Kavlock et al., 1996) in fish, the genetic factors involved in gonadal abnormality have received little attention. Although there are few mutant lines showing gonadal abnormalities e.g. mutant Zebrafish (Basten et al., 2013b), they only cover narrow aspects of reproductive biology.

Reported here is a naturally occurring testicular abnormality, that shows perturbation to multiple aspects of testicular development and function. This study examined 6111 carp from a single cohort, detecting 487 individuals with Jelly-like gonad condition (JGC). This sample size is significantly larger than other published studies on naturally occurring gonadal abnormalities in vertebrates. Prior to undertaking this investigation, little was known about causes of the mass gonadal abnormality or its consequences to individual health and reproductive performance. Collectively, this study employed a combination of scientific approaches to address the cause and effect of JGC carp that causes male infertility in a lacustrine population of inbred carp.

5.1. Etiology and prevalence of JGC:

The detailed epidemiological study of JGC in Lake Sorell revealed species and sex specificity of the condition, suggesting non-environmental etiology of the condition (Chapter 2). For example, gonadal abnormality originating from environmental pollution conceivably affect both sexes (Wiklund et al., 1996), and exhibits many morphological and categorical forms (e.g. aplasia, fusions, constrictions, compartmentations and asymmetries) (Bittner, 2009) including occurrence of intersex populations (Jobling et al., 1998) that are not restricted to a single sex. In the case of JGC it was not possible to distinguish any such shared abnormalities, between the sexes, apart from the level of JGC severity in just the males. Collectively, the pristine nature of Lake Sorell, sex- and species- specificity, non-activation of immune pathway or an absence of bacteria in histological sections with no other external sign (i.e. lesion) of disease suggests a genetic etiology of JGC. Effort to culture bacteria from JGC tissue were mostly unsuccessful except for a few (≥6) colonies in several plates. Molecular profiling of those bacteria indicated them to be of laboratory and environmental contamination (data not presented). Similarly, we failed to observe the presence of inclusion bodies which is common phenomenon for most cases of viral infection (Arakawa et al., 1989; Giraldo et al., 1972; Isshiki et al., 2001; Park et al., 1998). Conceivably also, a viral infection will affect both sexes with probable spread to other visceral organs (Ahne et al., 2002; Jung and Miyazaki, 1995; Mulcahy et al., 1983). It is therefore unlikely that JGC is caused by an infective agent such as a bacterium or virus. Moreover, no other parasitic infections were obvious. As the Lake Sorell carp population originated from a handful of fish that escaped from live bait used by the anglers (Patil et al., 2015), the degree of inbreeding would probably be extremely high and this may have predominantly contributed to the development of the condition.

Overall, long term observation of a single cohort of wild fish provides a unique understanding of timeseries data that precisely indicates the relationship of the JGC condition to maturity (Chapter 2). Although maturity of the carp and their hybrids was previously implied to be related to gonadal abnormality, data was limited to a few individual cases (Chiba et al., 1979; Raidal et al., 2006). In contrast, an extensive carp eradication program in Lake Sorell enabled investigation of the gonadal condition in every fish that was caught from 2012 to 2017; making this frequency analysis unique and most comprehensive.

5.2: Morphological staging of JGC:

ImageJ has been considered as a powerful tool for biologists to analyse tissue and ultrastructural photographs (Papadopulos et al., 2007). While many of the modern image processing could employ machine intelligence to perform complex tasks (i.e. pattern analysis) in medical image processing, they are expensive and very challenging to use (for review see Duncan and Ayache, 2000). In comparison, ImageJ is a freeware with many of the plugins available for specific tasks (Girish and Vijayalakshmi, 2004; Rasband, 2008). The application of image analysis software to calculate affected tissue has been previously used (Haase et al., 1996), and was successfully employed for quantitative analyses to determine JGC severity (Chapter 2).

Based on gross morphology the severity of the JGC condition could be divided into 4 distinct categories; stage 1(low), stage2 (mild), stage 3 (medium) and stage 4 (complete). All the downstream analyses (i.e. histology, TUNEL staining, hormonal assay and RNAseq) that were performed using the stages of JGC as a criteria, revealed unique characteristics for each severity category. For example, histological observation indicated that Stage 1 is characterized by uncontrolled proliferation of Sertoli cells with disease progressing to unaffected areas, Stage 2 is marked by onset of cell death, Stage 3 is

marked by the presence of large vacuoles with mass cell death and Stage 4 is marked by an almost cell free, fluid filled, vacuolated organ. Similarly, TUNEL staining of the JGC stages revealed an increasing propensity of cell death. Analysis of circulating hormone also revealed distinct hormone profiles for each of the categories. Collectively, results of this study suggested that the criteria set for JGC severity analysis were not only suitable but can be used for future JGC research.

5.3. Potential of JGC as a cancer model:

Cancer and neoplasm of gonads are considered major causes of reproductive failure (Eble et al., 2004). Specifically, cancer in testicular tissue has been increasing at an annual rate of 2.3-5.3% in humans (Adami et al., 1994; Huyghe et al., 2003). The bulk of the reported cases pertain to germ cell cancer, with non-germ cell testicular cancer comprising c.5-10% of the total testicular cancer (Chapter 2). As a frequent disease, germ cell cancer has been rigorously investigated (Oosterhuis and Looijenga, 2005; Rosenberg et al., 1998; Sandberg et al., 1996) with model organisms available for almost all subtypes (Neumann et al., 2011; Oosterhuis and Looijenga, 2005).

Model organisms are essential to understand many critical and shared features as well as the pharmacodynamics and pathobiology of many cancer forms (Arap et al., 1998; Couch, 1995; Olive et al., 2009; Sharpless and DePinho, 2006). For example, zebrafish line having mutation in *lrrc50* serves as a model for seminoma (Basten et al., 2013b), inbred mouse strain-129 serve as a model for teratomas (Stevens Jr and Little, 1954; Stevens, 1970; Walt et al., 1993); however such examples are rare for non-germ testicular cancer. To date the only model organism/system for non-germ cell testicular cancer are the transgenic lines of mouse (Matzuk et al., 1992; Pangas et al., 2008; Peschon et al., 1992), however they all have limitations for further study. For example, they were not male specific (Matzuk et al., 1992) and the animal could not be maintained due to reduced

fertility (Peschon et al., 1992). Moreover, generation of transgenic lines require prior knowledge of the candidate/target genes. In contrast, this study examined a naturally occurring gonadal abnormality in carp that occurs at high prevalence and thus may provide an unique opportunity to understand what appears to be a non-germ cell cancer of Sertoli cell origin. Specifically, histopathological observations of the condition clearly demonstrated uncontrolled proliferation of the Sertoli cells (Chapter 2) that eventually rendered the animal sterile (Chapter 3). Similarly, transcriptomic analysis using JGC and control carp demonstrated the upregulation of several testicular cancer markers i.e. *sall4* (Cao et al., 2009), *cx43* (Chevallier et al., 2013), *anapc11*(Chan et al., 2001) and *hormad1* (Yao et al., 2014) indicating the condition is likely cancerous in the early stages (Chapter 4). However, massive cell death in the later stages of JGC makes it distinct from commonly observed cancer scenarios. Regardless of minor dissimilarities with commonly known non-germ cell cancer (i.e. absence of metastasis and cell death) (Dilworth et al., 1991); this study, indicates the occurrence of JGC carp could be a suitable model for studying health and therapeutic implications of non-germ cell cancer of the testes.

5.4. Sterility and growth – implications for aquaculture and pest management:

This study found that JGC is limited to male carp and eventually leads to sterility in affected individuals (Chapter1 and 2). Field data indicated a statistically significant relationship between the JGC and fishing year. This relationship was expected as the frequency of JGC increased over the periods of successive fishing years (Chapter 2). Furthermore, there was a significant growth difference between normal and JGC fish (Chapter 3). Overall, JGC fish exhibited 6-15% more body weight and were 3.7-7.5% longer than the rest of their unaffected cohorts. Analysis of mark recaptured data

indicated that this accelerated growth occurred after the initiation of the JGC condition. The probable reasons for the increased growth have been discussed in Chapter 3.

Normally, fish with gonadal anomaly display fatigue and physical malformation (Hawkins et al., 1996; Raidal et al., 2006). For example, hybrids of carp with gonadal abnormality develop multinodular masses that results in an abdominal swelling and impaired movement (Down and Leatherland, 1989). In advanced cases, multinodular non-encapsulated firm masses can fill the entire coelomic cavity with invasion into the liver lobes and intestinal loops (Sirri et al., 2010). However, in JGC carp, no JGC related mortality was observed. In fact, JGC fish grew faster and the condition of the fish remained unchanged compared to normal males (Chapter 3) indicating the JGC fish had proportional, but accelerated growth compared to unaffected male cohorts. Therefore, the aquaculture potential for JGC fish appears promising. However, as JGC fish accommodated an abnormal gonad, the consumer acceptance of the JGC fish may be compromised. This is less likely, as the only affected part of the fish is the testis with no adverse effect to other organs. Additionally, while the female gonads have a market value as fish roe (Bledsoe et al., 2003; Mol and Turan, 2008), the use of testis as a food is limited. Therefore, JGC fish (males) may have aquaculture potential, that needs further exploration.

Both histological and physiological observations clearly demonstrated a sudden arrest of spermatogenesis (Chapter 2), with concomitant reduction in milt quality (Chapter 3) of JGC fish. Moreover, computer assisted sperm analysis (CASA) of the affected and unaffected milt indicated that mean sperm velocity of JGC fish was significantly lower. Collectively, this study revealed that the JGC fish are almost sterile especially in an advanced condition. Whilst the absolute mechanism for spermatogenic arrest and infertility in the JGC testis is not clear. Transcriptomic analysis indicated that several key

genes i.e. *spaca9*, *tex36* (Cai et al., 2017), *ropn1l* (Fiedler et al., 2013) and *spata4* (Jiang et al., 2015) involved in male fertility and spermatozoa capacitation were downregulated (Chapter 4). While the molecular analysis supported the physiological observations leading to sterility, it is not yet clear as to which master gene is causing the arrest of spermatogenesis in JGC. Further research into key differentially expressed genes (Chapter 4) could shed more light on the observed testicular infertility.

From another applied perspective, sterility of the fish is desirable in large scale management of feral fish populations. One classical example is the usage of 'Judas' carp in carp eradication programs (Bajer et al., 2011; Diggle et al., 2012; Taylor et al., 2012). Although this technique has proven successful in the past (Diggle et al., 2012), the risk of the tagged animal spawning possess a serious threat for the management activity (Patil et al., 2015). In contrast, as demonstrated in this study the deployment of JGC carp as sterile Judas fish can effectively mitigate the recruitment risk, whilst assisting eradication efforts (IFS report 2013). More broadly, elucidating the genetic drivers (genes) of sterility in the JGC fish could assist in developing species-specific genetic carp control options. For example, it has been suggested that genetic control of carp through Immuno-contraception could be a valuable method for carp control (Hinds and Pech, 1996). This method is dependent on selective disruption of reproductive system through targeting gonad specific gene or gene product. Although, vitellogenin has been suggested as a target for ovary, the testis specific antigen is yet to be developed (Hinds and Pech, 1996). While, the mode of reproduction is conserved among the species, targeting a protein (i.e. vitellogenin) or pathway (hypothalamic-pituitary pathway) that is conserved between the species could be detrimental to native species, if they interbreed. Therefore, identification of candidates for a carp specific testicular sterility, could be valuable for assisting immune-contraception or related sex manipulation approaches.

5.5: JGC is a potential system for investigating cell death:

The study of cell death is unique and complex in nature, as this often requires triggering the pathways (apoptosis, autophagy and necrosis) through starvation, steroid treatment and chemotherapy (Adams, 2003). Such stress could create a bias in cellular environment compromising the outcome. Histopathological observation aided by the TUNEL assay (Chapter 2) indicated that the gross degeneration of JGC testes is characterised by cell death. There are three different modes of cell death–apoptosis, autophagy and necroptosis. It was generally believed that the different modes of cell death are independent (Tsujimoto and Shimizu, 2005), however, a recent research review suggested them to be interlinked (Thorburn, 2008). The gene expression study suggested that the JGC tissue supports the latest theories of cell death (Chapter 4). Hence this (JGC carp) could be a valuable system for further study on cell death and the underpinning mechanisms.

5.6: Genetic drivers of JGC development:

There are several studies documenting gonadal abnormality in fish (Fitzsimons and Cairns, 2000; Ishikawa et al., 1976; Jobling et al., 1998; Mikaelian et al., 2002; Wiklund et al., 1996), but descriptions were limited to frequency distribution and histology and no data for gene expression can be found. The study of mass gonadal neoplasia in carp and their hybrids from the Great Lakes, North America provides a detailed basis for normal and abnormal testicular condition with endocrine and hormonal response (Down, 1984; Down and Leatherland, 1989; Down et al., 1988). However, genetic drivers influencing the condition remained unexplored simply because of the lack of technology. In contrast, this study took advantage of the latest developments in transcriptome analyses to examine the global differential expression of genes in JGC and normal gonads. Such application of gene expression technology between normal and abnormal testicular tissue using state of the art next-generation RNA sequencing technology has not been applied for this

purpose. An earlier study on gonadal abnormality in whitefish (*Coregonus* spp.) from Lake Thun, Switzerland (Bittner, 2009) was the first attempt to employ the transcriptomic approach to understand the genetic contribution in development of the condition. However, despite the large number of genes arrayed, only one immune gene was found differentially expressed between the individuals with no association to the abnormality investigated (Bittner et al., 2011; Bogdal et al., 2009). This can be attributed to limitations of micro-array technology. Generally, microarray can only screen for known genes that already exist in the database, precluding discovery of novel genes that may be associated with the disease or condition (Larsson et al., 2006). Microarrays also suffer the limitation of identifying splice variants (Romero et al., 2018) when compared to RNAseq (Oshlack et al., 2010; Trapnell et al., 2012; Wang et al., 2009). Significantly, RNAseq is a revolutionary technology that does not suffer from complications with background noise and cross hybridization like microarrays (Wang et al., 2009). For example, experiments using RNAseq have discovered of large number of uniquely transcribed regions when applied to many species e.g. *A. thaliana* (Lister et al., 2008), *S. cerevisiae* (Nagalakshmi et al., 2008), *S. pombe* (Wilhelm et al., 2008), the mouse (Cloonan et al., 2008; Mortazavi et al., 2008; Roberts et al., 2011), and humans (Hangauer et al., 2013; Morin et al., 2008).

This study represents the first to document through next-generation RNAseq (Chapter 4) total transcriptomic response (including long non-coding RNA but excluding miRNA) associated with an abnormal fish testis. The study identified a large number (n=7,129) of differentially expressed genes (DEGs) not only indicating substantial changes in testicular state of the affected fish but also confirming the spatial and temporal association of these genes in teleost testicular tissue.

Genomically, the common carp is considered a complex organism that has evolved from allotetraploidization (Xu et al., 2014) and many of the duplicated genes are still expressed

albeit with functional sub-specialisation (Barney et al., 2008; Larhammar and Risinger, 1994). Briefly, it contains twice as many chromosomes (2n=100) compared with other members of the family Cyprinidae e.g. for zebrafish 2n=50 (Postlethwait et al., 1998). As common carp has a complex genome (Xu et al., 2014) with very high DNA content (David et al., 2003), the genetic map and annotation are still incomplete compared to humans or Zebrafish. Despite the limitation of genome annotation, this study successfully analysed gene expression analysis.

There is a strong evidence from the analysis that among the DEGs, 40 genes were directly associated with the condition. As presented in the Chapter 4, 26 of the top 40 genes are completely unknown to recent knowledge; making this study uniquely successful in identifying novel-candidate genes/marker for gonadal abnormality. However, a future characterization of the unknown genes is required to discern their roles in the development and regulation of JGC condition in common carp. The next obvious challenge is to target those complex unannotated regions/mRNA/ncRNAs in order to identify and track the expression changes including the detection of spliced variants in JGC and multiple tissue forms. Comparative cross species studies may also be necessary to infer the conserved role of these novel genes. Overall, candidate identified through transcriptomic profiling described in this study will not only open up a new door for future research of the candidate gene approach to understand gonadal abnormality, spermatogenesis, apoptosis, and cancer in general, but also increase the likelihood of discovering a master regulator for testicular dynamics and related processes.

Since multiple factors alter the gene expression pattern in an individual, successful application of RNAseq technology critically depends on the interpretation of the differentially expressed genes in appropriate pathways (Khatri et al., 2012; Subramanian et al., 2005). For example, the expression of many genes may strongly differ over time

between season (Fangue et al., 2006; Yang and Loopstra, 2005), sex (Carrel and Willard, 2005; Ranz et al., 2003), and tissue location (Barney et al., 2008; Pickrell et al., 2010). Hence, many of the observed differentially expressed genes could be explained by natural variation between individuals (despite the usages of biological replicates). However, a systematic pathway analysis of the DEGs can normalise and overcome such limitations (Gu et al., 2012).This study not only classified the DEGs into corresponding pathways, but also tested the significance of each of the pathways with regards to the development of JGC occurrence using the 'Two sided hypergeometric test' with Bonferroni stepdown normalization. The pathway analyses presented in this thesis investigated a number of databases in parallel (KEGG; Go-Molecular function, cellular component, biological process, Immune system; Interpro-protein domain; Reactome among others) rather than focussing on a single database. This was done to ensure the complete detection of biological processes altered in JGC gonad.

Pathway analyses not only provided a clear insight into the mechanism of JGC condition but also reflected the morphological and histopathological observation that were observed earlier (Chapter 2 and 3) i.e. cell death, cancer, arrest of spermatogenesis, structural disruption, and vacuole production.

5.7. Conclusion

This thesis employed a multidisciplinary approach to address a unique gonadal abnormality that occurs in an inbred carp population. Briefly, the study provides a morphological and anatomical description of the condition that exhibits increased severity with maturity of the affected animals. The abnormality had no relationship with environmental variables, such as capture site and lacked pathological symptoms associated with bacterial or viral infections. Instead the condition appears cancerous with a genetic predisposition. The outcomes suggest that this sub-population of carp may be a

good system to assist understanding of non-germ cell cancer, endocrine disruption and the mechanisms of cell death. Moreover, as JGC fish displays improved growth, the aquaculture potential of JGC fish is promising. This improved growth in combination with likely sterility could have multiple applications including in generating sterile Judas carp (Patil et al 2015, IFS Report 2013) and identifying candidate genes for manipulating sex ratios for large scale management of their pest populations. From an aquaculture perspective, a key bottleneck could be an inability of JGC fish to produce gametes for propagation of seed. This study presents the first time employment of next generation whole transcriptome sequencing to uncover the molecular secret behind a novel gonadal abnormality. Data generated by the RNAseq analyses are currently being investigated to characterise 'novel' genes that may have a role in reproductive biology as well as for isolation of genomic markers to assist breed selection.

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Appendix A

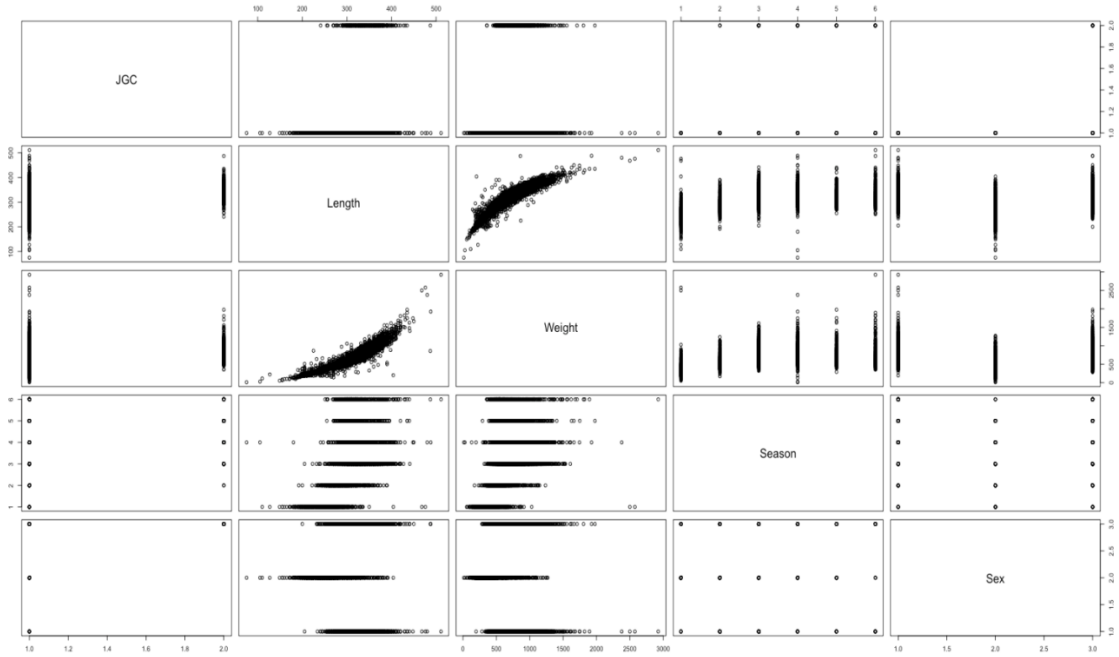


Fig A.1 schematic relationship between the predictors of JGC regression.

Table A.1 Regression analysis

Coefficients	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-1.06E+01	1.38E+00	-7.709	1.27E-14
Sex M	4.84E+00	3.91E-01	12.377	< 2e-16
Weight	2.48E-03	2.89E-04	8.597	< 2e-16
Length	1.86E-02	2.08E-03	8.931	< 2e-16
Season 13/14	1.62E+00	6.18E-01	2.626	0.00864
Season 14/15	2.08E+00	6.35E-01	3.276	0.00105
Season15/16	3.12E+00	6.48E-01	4.818	1.45E-06
Season16/17	3.82E+00	6.52E-01	5.852	4.85E-09
Month11	4.80E-01	1.14E+00	0.423	0.67263
Month9	-8.70E-01	1.35E+00	-0.643	5.20E-01
Month8	9.35E-01	1.41E+00	0.663	0.50762
Month7	-3.88E-01	1.55E+00	-0.25	0.80276
Month6	-9.19E-01	1.55E+00	-0.595	5.52E-01
Month4	-1.64E+00	1.53E+00	-1.07	2.85E-01
Month3	-7.32E-01	1.15E+00	-0.634	0.52608
Month2	7.77E-02	1.14E+00	0.068	0.94565
Month1	3.21E-02	1.14E+00	0.028	0.97746
Month10	2.46E-01	1.15E+00	0.214	0.83067
Month12	1.20E-01	1.14E+00	0.106	0.9159
Mirror	1.15E-01	2.36E-01	0.486	0.62729
Method4.75	5.69E-01	3.97E-01	1.432	0.15221
Method4.75c	4.93E-01	4.39E-01	1.123	0.26149

Method bcn	1.23E+00	1.42E+00	0.865	0.3873
Method bfc	-1.57E+01	2.40E+03	-0.007	0.99477
Method bfn	-3.89E-01	3.26E-01	-1.193	0.23274
Method dfyke	-1.19E+01	2.40E+03	-0.005	0.99605
Method eb	-2.59E-01	7.49E-01	-0.346	0.72926
Method Eel Fisherman	-1.32E+01	4.78E+02	-0.028	0.97791
Methodm4	-1.40E+01	1.46E+03	-0.01	0.99233
Method Main Drain F	1.684e+01	2.40E+03	0.007	0.9944
Methodmm4	1.83E-01	2.08E-01	0.876	0.38117
Methodmm4B	1.03E+00	1.10E+00	0.938	0.34841
Methodmm4t	1.14E-01	3.91E-01	0.293	0.76988
Methodmm4tb	2.82E+00	1.69E+00	1.663	0.09632
Methodmm4tw	1.43E+00	7.74E-01	1.847	0.06475
Methodmm5	4.69E-01	2.19E-01	2.14	0.03239
Methodmmtw	1.71E+01	2.40E+03	0.007	0.99431
Method	-3.07E-01	3.31E-01	-0.926	0.35448
Methodn4.5	-1.09E+01	2.40E+03	-0.005	0.99637
Methodn4.5b	-1.21E+01	2.40E+03	-0.005	0.99598
Methodn4.5w	2.07E+00	2.35E+00	0.881	0.37855
Methodn4.75	2.63E-02	2.36E-01	0.111	0.91124
Methodn4.75c	4.38E-01	3.53E-01	1.24	0.21498
Methodn4b	-1.99E+00	1.21E+00	-1.643	0.1003
Methodn4c	-1.06E+00	7.54E-01	-1.404	0.16029
Methodn4t	-1.00E+00	6.40E-01	-1.567	0.11708
Methodn4tw	-8.85E-02	1.26E+00	-0.07	0.94411
Methodn4w	1.54E+01	2.40E+03	0.006	0.99489
Methodn5	7.32E-01	2.40E-01	3.052	0.00228
Methodn5b	-6.91E-02	6.61E-01	-0.105	0.91668
Methodn5c	-1.26E+00	1.45E+00	-0.872	0.38331
Methodn5h	5.82E+00	1.39E+00	4.173	3.00E-05
Methodpbc	-1.24E+01	2.40E+03	-0.005	0.99589
Methodpen	-1.44E+01	1.25E+03	-0.011	0.99083
Methodrfn	-1.41E+01	2.40E+03	-0.006	0.99532
Methodsfn	-2.29E-01	6.46E-01	-0.355	0.72257
MethodSTFN	1.47E+00	1.29E+00	1.134	0.25667
Methodtmm4	1.84E+01	2.40E+03	0.008	0.99387

Appendix B

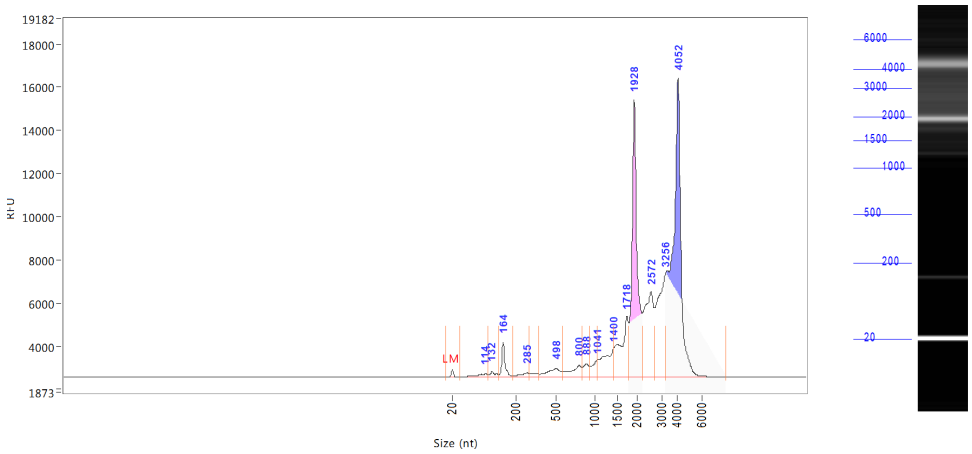


Fig B.1 Representative quality control result of RNA sample (RIN score above 7). Note: Two sharp picks (16s and 18s) confirmed high integrity of the total RNA samples. Corresponding gel picture on the right.

Table B.1 Raw read statistics of RNASeq data.

Sample ID	Total read bases (bp)	Total reads	GC(%)	AT(%)	Q20(%)	Q30(%)
1_JG	9,379,275,312	92,864,112	49.433	50.57	96.886	94.717
2_JG	10,177,988,766	100,772,166	47.355	52.65	97.097	95.083
MC_11	8,912,708,842	88,244,642	50.133	49.87	97.043	94.941
MC_16	9,761,837,052	96,651,852	48.249	51.75	96.931	94.79

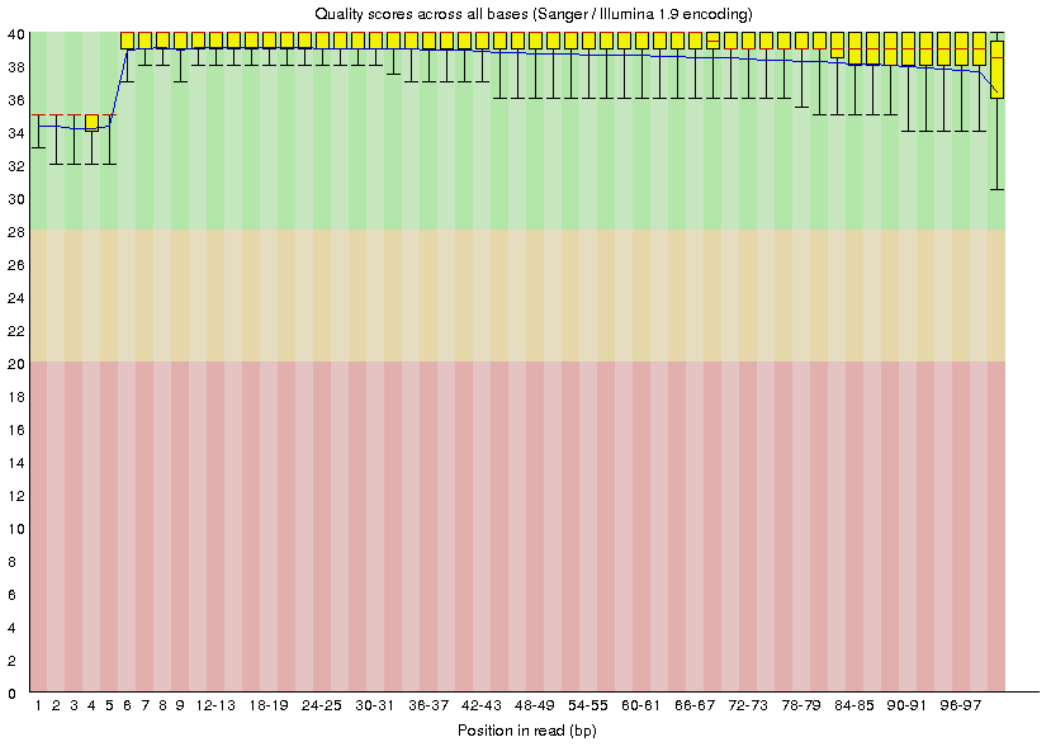


Fig B.2 FastQC result on filtered data.

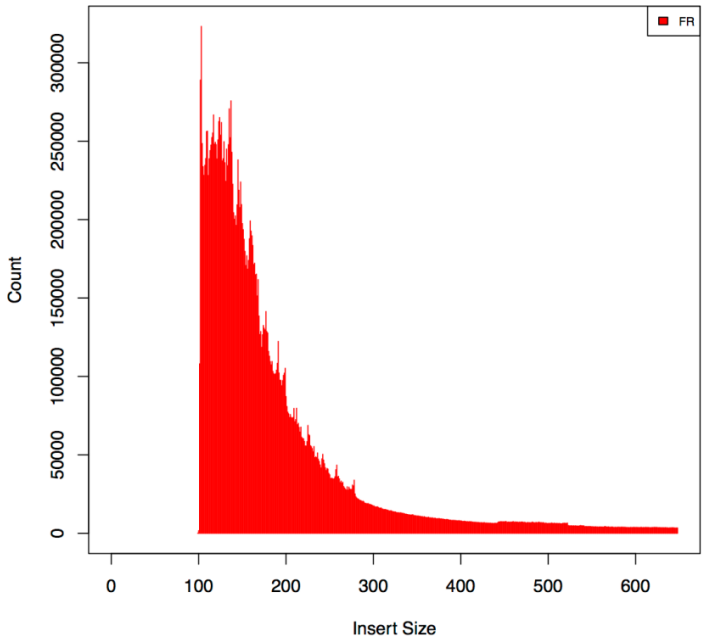


Fig B.3 An overall distribution of the insert size for the RNAseq experiment.

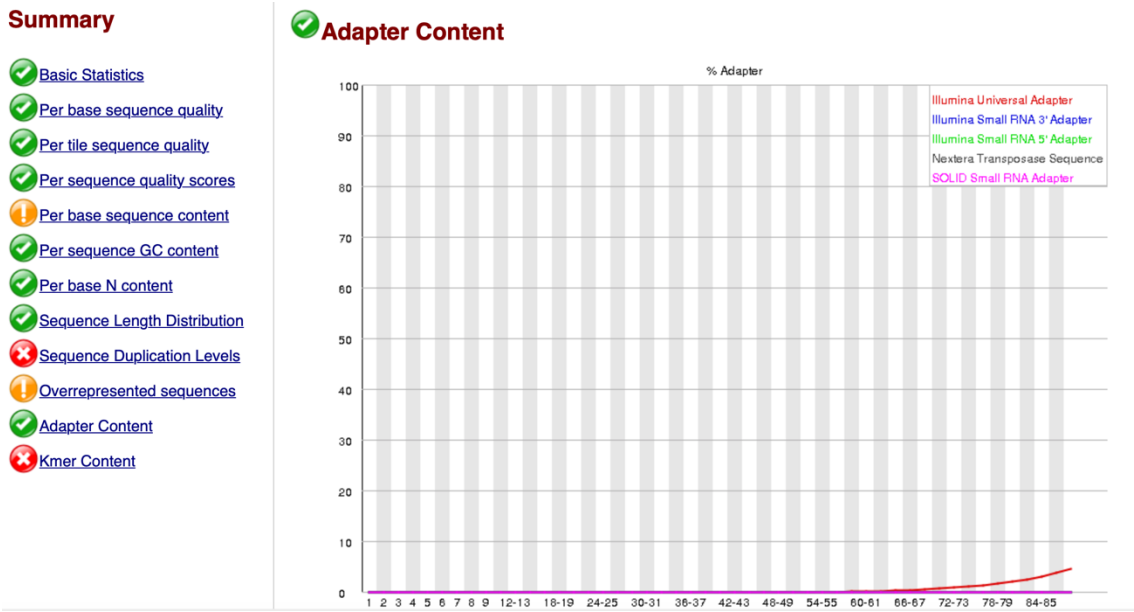
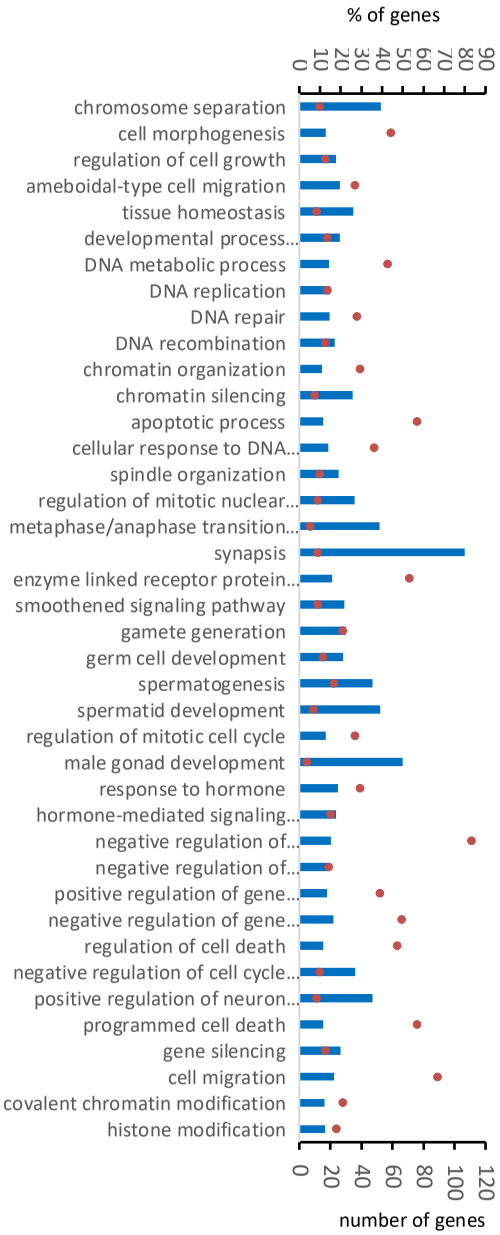
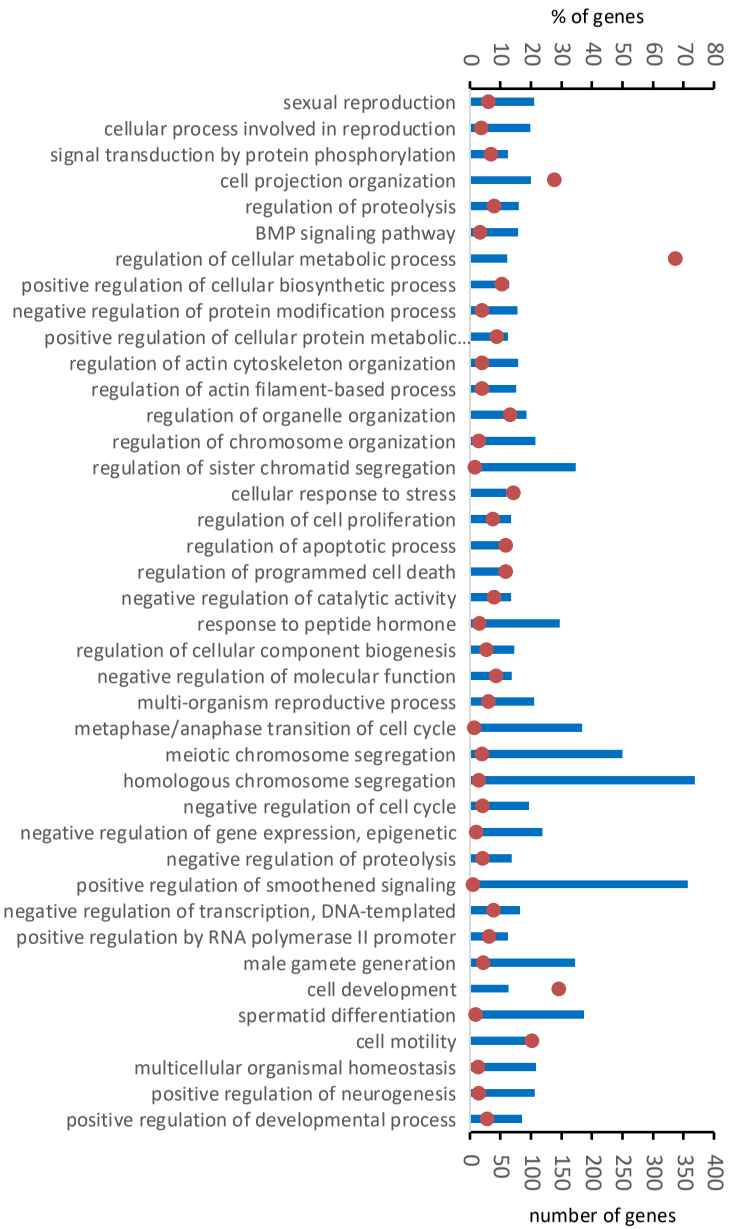


Fig B.4: A summary of the quality parameter along with the adapter content of a representative sample.

Table B.2 Alignment summary of the reads against reference genome.

Sample ID	Input reads (in pairs)	Mapped reads	Mapping rate	Multiple alignments
1_JG	41088214	29766340	72.4%	7816175 (26.3%)
2_JG	45169763	34445646	76.3%	9048452(26.3%)
MC_11	39320302	30691954	78.1%	8902538 (29.0%)
MC_16	42795238	32807894	76.7%	9462705 (28.8%)


% of genes
0 10 20 30 40 50 60 70 80 90
chromosome separation
cell morphogenesis
regulation of cell growth
ameboidal-type cell migration
tissue homeostasis
developmental process...
DNA metabolic process
DNA replication
DNA repair
DNA recombination
chromatin organization
chromatin silencing
apoptotic process
cellular response to DNA...
spindle organization
regulation of mitotic nuclear...
metaphase/anaphase transition...
synapsis
enzyme linked receptor protein...
smoothened signaling pathway
gamete generation
germ cell development
spermatogenesis
spermatid development
regulation of mitotic cell cycle
male gonad development
response to hormone
hormone-mediated signaling...
negative regulation of...
negative regulation of...
positive regulation of gene...
negative regulation of gene...
regulation of cell death
negative regulation of cell cycle...
positive regulation of neuron...
programmed cell death
gene silencing
cell migration
covalent chromatin modification
histone modification
0 20 40 60 80 100 120
number of genes


% of genes
0 10 20 30 40 50 60 70 80
sexual reproduction
cellular process involved in reproduction
signal transduction by protein phosphorylation
cell projection organization
regulation of proteolysis
BMP signaling pathway
regulation of cellular metabolic process
positive regulation of cellular biosynthetic process
negative regulation of protein modification process
positive regulation of cellular protein metabolic...
regulation of actin cytoskeleton organization
regulation of actin filament-based process
regulation of organelle organization
regulation of chromosome organization
regulation of sister chromatid segregation
cellular response to stress
regulation of cell proliferation
regulation of apoptotic process
regulation of programmed cell death
negative regulation of catalytic activity
response to peptide hormone
regulation of cellular component biogenesis
negative regulation of molecular function
multi-organism reproductive process
metaphase/anaphase transition of cell cycle
meiotic chromosome segregation
homologous chromosome segregation
negative regulation of cell cycle
negative regulation of gene expression, epigenetic
negative regulation of proteolysis
positive regulation of smoothened signaling
negative regulation of transcription, DNA-templated
positive regulation by RNA polymerase II promoter
male gamete generation
cell development
spermatid differentiation
cell motility
multicellular organismal homeostasis
positive regulation of neurogenesis
positive regulation of developmental process
0 50 100 150 200 250 300 350 400
number of genes

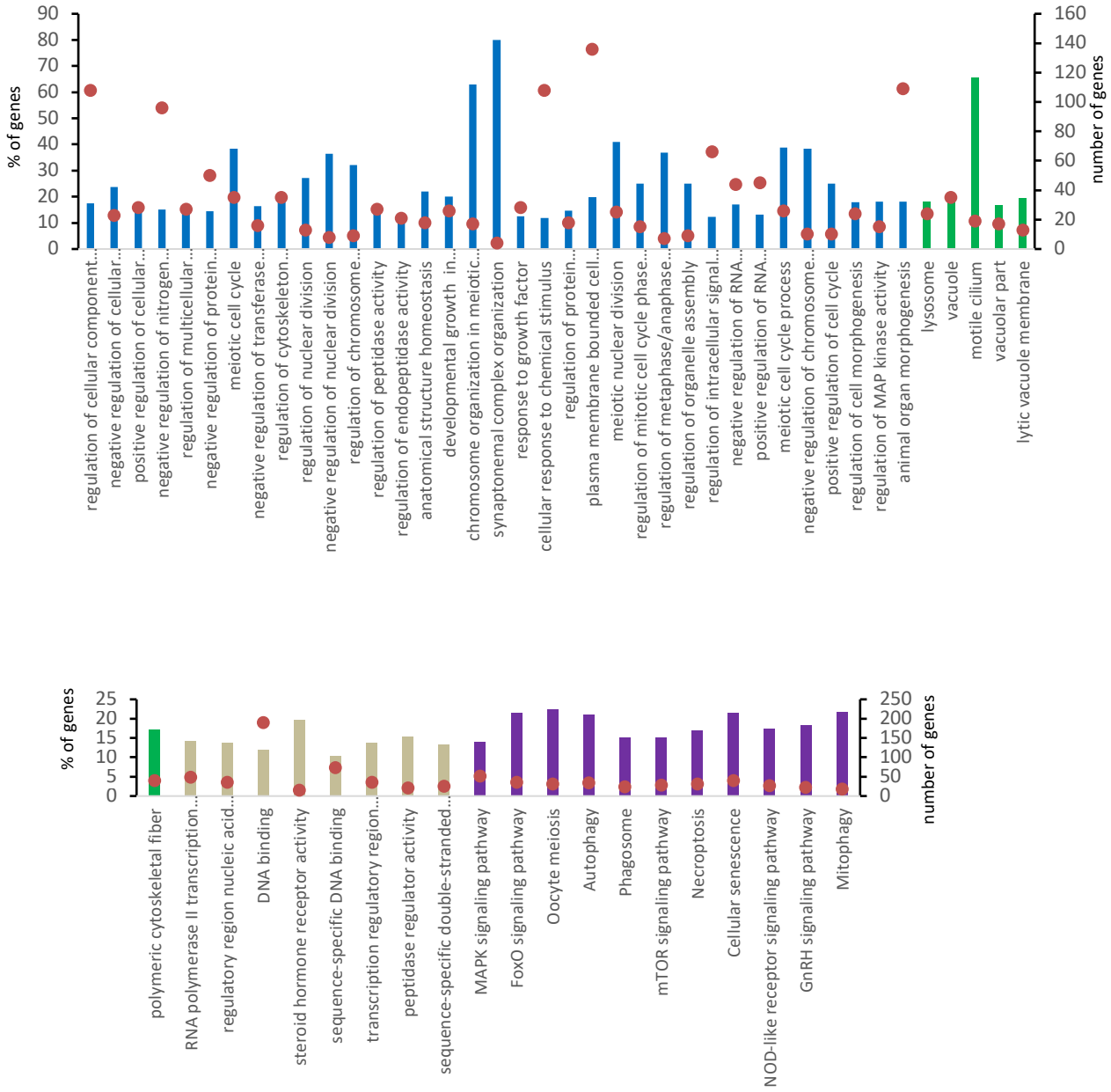


Fig B.5.1-5. Gene ontology classification of unigenes representing the number of genes in each category and the percentage of the genes within the pathway. Pathways were enriched from four ontology sources, namely GO-Biological process, GO-Cellular Component, GO-Molecular Function and KEGG pathway. Note: the percentages are represented in a bar diagram (scale left) and the number of genes (scale right) are represented with dots.

Table B.3 A list of overrepresented terms by ontology analysis of differentially expressed genes.

Number	GOID	GOTerm	Ontology Source	Term PValue
1	GO:0051304	chromosome separation	GO_BiologicalProcess	8.80E-53
2	GO:0000902	cell morphogenesis	GO_BiologicalProcess	2.07E-42
3	GO:0000981	RNA polymerase II transcription factor activity, sequence-specific DNA binding	GO_MolecularFunction	1.85E-41
4	GO:0001067	regulatory region nucleic acid binding	GO_MolecularFunction	1.67E-27
5	GO:0001558	regulation of cell growth	GO_BiologicalProcess	2.05E-27
6	GO:0001667	ameboidal-type cell migration	GO_BiologicalProcess	2.69E-26
7	GO:0001894	tissue homeostasis	GO_BiologicalProcess	7.41E-23
8	GO:0003006	developmental process involved in reproduction	GO_BiologicalProcess	2.14E-22
9	GO:0003677	DNA binding	GO_MolecularFunction	3.02E-21
10	GO:0003707	steroid hormone receptor activity	GO_MolecularFunction	2.24E-19
11	GO:0005764	lysosome	GO_CellularComponent	2.42E-19
12	GO:0005773	vacuole	GO_CellularComponent	2.46E-17
13	GO:0006259	DNA metabolic process	GO_BiologicalProcess	2.34E-15
14	GO:0006260	DNA replication	GO_BiologicalProcess-	5.28E-15
15	GO:0006281	DNA repair	GO_BiologicalProcess	9.69E-15
16	GO:0006310	DNA recombination	GO_BiologicalProcess	3.94E-14
17	GO:0006325	chromatin organization	GO_BiologicalProcess	4.64E-14
18	GO:0006342	chromatin silencing	GO_BiologicalProcess	7.07E-14
19	GO:0006915	apoptotic process	GO_BiologicalProcess	4.02E-13
20	GO:0006974	cellular response to DNA damage stimulus	GO_BiologicalProcess	4.77E-13
21	GO:0007051	spindle organization	GO_BiologicalProcess	4.13E-12
22	GO:0007088	regulation of mitotic nuclear division	GO_BiologicalProcess	5.87E-12
23	GO:0007091	metaphase/anaphase transition of mitotic cell cycle	GO_BiologicalProcess	7.47E-11
24	GO:0007129	synapsis	GO_BiologicalProcess	1.03E-10
25	GO:0007167	enzyme linked receptor protein signaling pathway	GO_BiologicalProcess	1.05E-10
26	GO:0007224	smoothened signaling pathway	GO_BiologicalProcess	2.21E-10
27	GO:0007276	gamete generation	GO_BiologicalProcess	5.63E-10
28	GO:0007281	germ cell development	GO_BiologicalProcess	7.20E-10
29	GO:0007283	spermatogenesis	GO_BiologicalProcess	7.70E-10
30	GO:0007286	spermatid development	GO_BiologicalProcess	8.16E-10
31	GO:0007346	regulation of mitotic cell cycle	GO_BiologicalProcess	1.53E-09
32	GO:0008584	male gonad development	GO_BiologicalProcess	1.86E-09
33	GO:0009725	response to hormone	GO_BiologicalProcess	5.37E-09
34	GO:0009755	hormone-mediated signaling pathway	GO_BiologicalProcess	5.77E-09
35	GO:0009892	negative regulation of metabolic process	GO_BiologicalProcess	8.26E-09
36	GO:0010466	negative regulation of peptidase activity	GO_BiologicalProcess	1.18E-08
37	GO:0010628	positive regulation of gene expression	GO_BiologicalProcess	1.32E-08
38	GO:0010629	negative regulation of gene expression	GO_BiologicalProcess	1.48E-08
39	GO:0010941	regulation of cell death	GO_BiologicalProcess	1.48E-08
40	GO:0010948	negative regulation of cell cycle process	GO_BiologicalProcess	1.52E-08
41	GO:0010976	positive regulation of neuron projection development	GO_BiologicalProcess	1.61E-08
42	GO:0012501	programmed cell death	GO_BiologicalProcess	1.71E-08
43	GO:0016458	gene silencing	GO_BiologicalProcess	1.74E-08
44	GO:0016477	cell migration	GO_BiologicalProcess	1.76E-08
45	GO:0016569	covalent chromatin modification	GO_BiologicalProcess	2.56E-08
46	GO:0016570	histone modification	GO_BiologicalProcess	2.83E-08
47	GO:0019953	sexual reproduction	GO_BiologicalProcess	3.31E-08
48	GO:0022412	cellular process involved in reproduction in multicellular organism	GO_BiologicalProcess	4.83E-08
49	GO:0023014	signal transduction by protein phosphorylation	GO_BiologicalProcess	5.84E-08
50	GO:0030030	cell projection organization	GO_BiologicalProcess	9.99E-08
51	GO:0030162	regulation of proteolysis	GO_BiologicalProcess	1.07E-07
52	GO:0030509	BMP signaling pathway	GO_BiologicalProcess	3.03E-07
53	GO:0031323	regulation of cellular metabolic process	GO_BiologicalProcess	3.57E-07
54	GO:0031328	positive regulation of cellular biosynthetic process	GO_BiologicalProcess	5.34E-07
55	GO:0031400	negative regulation of protein modification process	GO_BiologicalProcess	5.67E-07
56	GO:0031514	motile cilium	GO_CellularComponent	1.12E-06
57	GO:0032270	positive regulation of cellular protein metabolic process	GO_BiologicalProcess	1.55E-06
58	GO:0032956	regulation of actin cytoskeleton organization	GO_BiologicalProcess	1.87E-06
59	GO:0032970	regulation of actin filament-based process	GO_BiologicalProcess	2.56E-06
60	GO:0033043	regulation of organelle organization	GO_BiologicalProcess	2.84E-06
61	GO:0033044	regulation of chromosome organization	GO_BiologicalProcess	3.30E-06
62	GO:0033045	regulation of sister chromatid segregation	GO_BiologicalProcess	4.60E-06
63	GO:0033554	cellular response to stress	GO_BiologicalProcess	8.86E-06
64	GO:0042127	regulation of cell proliferation	GO_BiologicalProcess	1.03E-05
65	GO:0042981	regulation of apoptotic process	GO_BiologicalProcess	1.06E-05

66	GO:0043067	regulation of programmed cell death	GO_BiologicalProcess	1.18E-05
67	GO:0043086	negative regulation of catalytic activity	GO_BiologicalProcess	1.20E-05
68	GO:0043434	response to peptide hormone	GO_BiologicalProcess	1.54E-05
69	GO:0043565	sequence-specific DNA binding	GO_MolecularFunction	1.78E-05
70	GO:0044087	regulation of cellular component biogenesis	GO_BiologicalProcess	3.11E-05
71	GO:0044092	negative regulation of molecular function	GO_BiologicalProcess	3.33E-05
72	GO:0044212	transcription regulatory region DNA binding	GO_MolecularFunction	3.33E-05
73	GO:0044437	vacuolar part	GO_CellularComponent	3.74E-05
74	GO:0044703	multi-organism reproductive process	GO_BiologicalProcess	4.28E-05
75	GO:0044784	metaphase/anaphase transition of cell cycle	GO_BiologicalProcess	4.32E-05
76	GO:0045132	meiotic chromosome segregation	GO_BiologicalProcess	4.88E-05
77	GO:0045143	homologous chromosome segregation	GO_BiologicalProcess	8.04E-05
78	GO:0045786	negative regulation of cell cycle	GO_BiologicalProcess	8.40E-05
79	GO:0045814	negative regulation of gene expression, epigenetic	GO_BiologicalProcess	1.02E-04
80	GO:0045861	negative regulation of proteolysis	GO_BiologicalProcess	1.24E-04
81	GO:0045880	positive regulation of smoothened signaling pathway	GO_BiologicalProcess	1.38E-04
82	GO:0045892	negative regulation of transcription, DNA-templated	GO_BiologicalProcess	1.45E-04
83	GO:0045944	positive regulation of transcription from RNA polymerase II promoter	GO_BiologicalProcess	1.60E-04
84	GO:0048232	male gamete generation	GO_BiologicalProcess	2.06E-04
85	GO:0048468	cell development	GO_BiologicalProcess	2.20E-04
86	GO:0048515	spermatid differentiation	GO_BiologicalProcess	2.24E-04
87	GO:0048870	cell motility	GO_BiologicalProcess	3.76E-04
88	GO:0048871	multicellular organismal homeostasis	GO_BiologicalProcess	3.76E-04
89	GO:0050769	positive regulation of neurogenesis	GO_BiologicalProcess	4.95E-04
90	GO:0051094	positive regulation of developmental process	GO_BiologicalProcess	5.50E-04
91	GO:0051128	regulation of cellular component organization	GO_BiologicalProcess	5.70E-04
92	GO:0051129	negative regulation of cellular component organization	GO_BiologicalProcess	7.22E-04
93	GO:0051130	positive regulation of cellular component organization	GO_BiologicalProcess	7.22E-04
94	GO:0051172	negative regulation of nitrogen compound metabolic process	GO_BiologicalProcess	7.74E-04
95	GO:0051240	positive regulation of multicellular organismal process	GO_BiologicalProcess	8.12E-04
96	GO:0051248	negative regulation of protein metabolic process	GO_BiologicalProcess	9.20E-04
97	GO:0051321	meiotic cell cycle	GO_BiologicalProcess	0.001085844
98	GO:0051348	negative regulation of transferase activity	GO_BiologicalProcess	0.001208161
99	GO:0051493	regulation of cytoskeleton organization	GO_BiologicalProcess	0.001492621
100	GO:0051783	regulation of nuclear division	GO_BiologicalProcess	0.001730256
101	GO:0051784	negative regulation of nuclear division	GO_BiologicalProcess	0.001976655
102	GO:0051983	regulation of chromosome segregation	GO_BiologicalProcess	0.002218312
103	GO:0052547	regulation of peptidase activity	GO_BiologicalProcess	0.002517605
104	GO:0052548	regulation of endopeptidase activity	GO_BiologicalProcess	0.003030951
105	GO:0060249	anatomical structure homeostasis	GO_BiologicalProcess	0.003129575
106	GO:0060560	developmental growth involved in morphogenesis	GO_BiologicalProcess	0.003807369
107	GO:0061134	peptidase regulator activity	GO_MolecularFunction	0.003813064
108	GO:0070192	chromosome organization involved in meiotic cell cycle	GO_BiologicalProcess	0.003872702
109	GO:0070193	synaptonemal complex organization	GO_BiologicalProcess	0.004271565
110	GO:0070848	response to growth factor	GO_BiologicalProcess	0.00444497
111	GO:0070887	cellular response to chemical stimulus	GO_BiologicalProcess	0.004955174
112	GO:0071900	regulation of protein serine/threonine kinase activity	GO_BiologicalProcess	0.005096198
113	GO:0098852	lytic vacuole membrane	GO_CellularComponent	0.005218174
114	GO:0099512	supramolecular fiber	GO_CellularComponent	0.005686626
115	GO:0099513	polymeric cytoskeletal fiber	GO_CellularComponent	0.00580943
116	GO:0120036	plasma membrane bounded cell projection organization	GO_BiologicalProcess	0.005843572
117	GO:0140013	meiotic nuclear division	GO_BiologicalProcess	0.005979101
118	GO:1901990	regulation of mitotic cell cycle phase transition	GO_BiologicalProcess	0.007506797
119	GO:1902099	regulation of metaphase/anaphase transition of cell cycle	GO_BiologicalProcess	0.009805924
120	GO:1902115	regulation of organelle assembly	GO_BiologicalProcess	0.009909287
121	GO:1902531	regulation of intracellular signal transduction	GO_BiologicalProcess	0.010264276
122	GO:1902679	negative regulation of RNA biosynthetic process	GO_BiologicalProcess	0.010365676
123	GO:1902680	positive regulation of RNA biosynthetic process	GO_BiologicalProcess	0.011693099
124	GO:1903046	meiotic cell cycle process	GO_BiologicalProcess	0.012002785
125	GO:1990837	sequence-specific double-stranded DNA binding	GO_MolecularFunction	0.01506238
126	GO:2001251	negative regulation of chromosome organization	GO_BiologicalProcess	0.015896242
127	GO:0045787	positive regulation of cell cycle	GO_BiologicalProcess	0.036856296
128	GO:0022604	regulation of cell morphogenesis	GO_BiologicalProcess	0.045404728
129	GO:0043405	regulation of MAP kinase activity	GO_BiologicalProcess	0.046964586
130	GO:0010976	positive regulation of neuron projection development	GO_BiologicalProcess	0.046964369

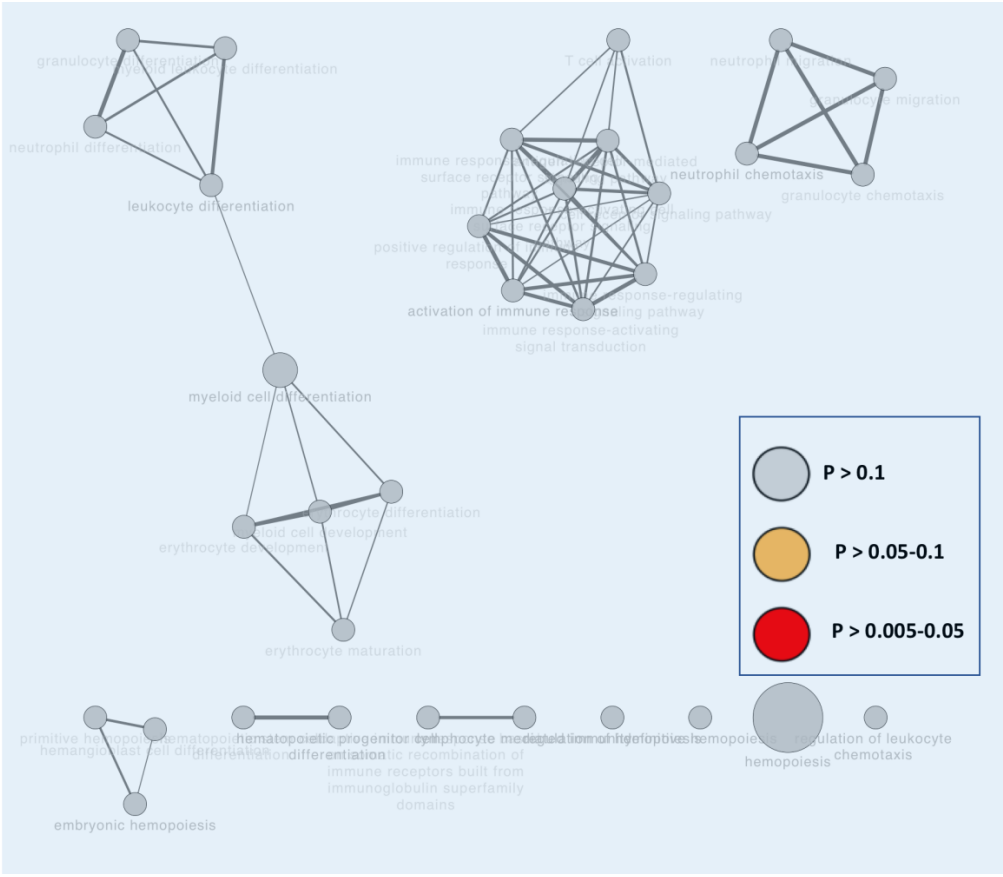


Fig B.6 Enrichment analysis of the DEGs showing non-significant results with immune reactivity.